TRIPLOIDÍA Y GINOGÉNESIS EN EL RODABALLO (*Scophthalmus maximus* L.). INDUCCIÓN, VERIFICACIÓN Y EFECTOS SOBRE EL CRECIMIENTO Y LA REPRODUCCIÓN

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Memoria presentada por Rosa M. Cal Rodríguez para optar al grado de Doctor en Ciencias Biológicas. Adscrita al Departamento de Ecología y Biología Animal de la Facultad de Ciencias del Mar de la Universidad de Vigo.

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El presente trabajo de investigación ha sido dirigido por el Dr. Francesc Piferrer Circuns, Investigador Científico del CSIC en el Institut de Ciències del Mar, en Barcelona, y tutelada por el Dr. Castor Guisande, Profesor Titular de la Facultad de Ciencias del Mar en la Universidad de Vigo.

Dr. Francesc Piferrer Circuns

“La fantasía abandonada de la razón produce
monstruos, pero unida a ella
es la madre de las artes”

Francisco Goya

A mis colegas Cas y Blanca,

porque se lo merecen.....

PREÁMBULO

En la acuicultura, el control de la reproducción de las especies en cautividad permite su domesticación y la aplicación de la tecnología adecuada para su mejora genética. En los sistemas actuales de producción del rodaballo (*Scophthalmus maximus* L.), los métodos utilizados para el control de la reproducción son ya habituales e incluyen la manipulación del fotoperiodo, la criopreservación del esperma, la inducción hormonal a la maduración y la aplicación de técnicas de mejora genética tradicional, tales como la selección fenotípica asistida en programas de cruzamientos. Estos métodos se ejercen sobre individuos adultos y permiten decidir "cuando" y "con quien" ha de efectuarse la reproducción.

Actualmente para muchas especies objeto de acuicultura se han desarrollado técnicas que permiten manipular la reproducción, decidiendo el "cómo", ya sea alterando el número de cromosomas, para obtener individuos estériles; manipulando la herencia de los progenitores, para obtener descendientes con herencia uniparental del progenitor elegido; aplicando tratamientos hormonales, para controlar la pubertad o producir poblaciones monosexo; y transgénesis, para modificar un carácter determinado que se trasmita a la descendencia. Estos métodos se ejercen sobre los gametos, los huevos recién fertilizados, los embriones en desarrollo o las larvas y los alevines.

El objetivo de este estudio fue desarrollar las técnicas de manipulación cromosómica para inducir la triploidía y la ginogénesis en el rodaballo, con el fin de producir lotes estériles y lotes todo hembras, respectivamente. Además, se verificó la bondad del método y las consecuencias biológicas de estas manipulaciones para determinar si los efectos observados cumplían las expectativas esperadas, de cara a explorar su posible aplicación en la acuicultura de esta especie.

Los experimentos descritos en este estudio se desarrollaron en el Módulo de Experiencias Biológicas del Instituto Español de Oceanografía de Vigo, desde 1996 a 2003, y fueron financiados con dos proyectos de investigación (CICYT, MAR95-1855 y UE-FEDER/DGESIC (1fd97-2404), cuyo investigador principal fue el Dr. Paulino Martínez de la Universidad de Santiago de Compostela.

El fruto de las investigaciones realizadas sobre la inducción, verificación y efectos de la triploidía y ginogénesis en el rodaballo se ha organizado en siete bloques definidos en torno a un aspecto particular. Estos bloques han generado las publicaciones que se detallan a continuación, que se reproducen como tal formando el corpus principal de esta memoria, la cual se complementa con una introducción y discusión general, seguida de las conclusiones y bibliografía consultada.

Piferrer, F., **Cal, R.M**., Álvarez-Blázquez, B., Sánchez, L. and Martínez, P. 2000. Induction of triploidy in the turbot (*Scophthalmus maximus*). I. Ploidy determination and the effects of cold shocks. **Aquaculture**, 188: 79-90.

Piferrer, F., **Cal, R.M**., Gómez, C., Bouza, C. and Martínez, P. 2003. Induction of triploidy in the turbot (*Scophthalmus maximus*). II**.** Effects of cold shock timing and induction of triploidy in a large volume of eggs. **Aquaculture**, 220: 821-831.

Castro, J., Bouza, C., Sánchez, L., **Cal, R.M**., Piferrer, F. and Martínez, P. 2003. Gynogenesis assessment by using microsatellite genetic markers in turbot (*Scophthalmus maximus)*. **Marine Biotechnology***,* 5: 584-592.

Piferrer, F., **Cal, R.M.,** Gómez, C., Álvarez-Blázquez, B., Castro, J. and Martínez, P. 2004. Induction of gynogenesis in the turbot (*Scophthalmus maximus*). Effects of UV-irradiation on sperm motility, the Hertwig effect, and viability during the first 6 months of age. **Aquaculture**, 238: 403-419.

Cal, R.M., Camacho, T., Vidal, S., Piferrer, F. and Guitián, F. J. 2005. Effect of triploidy on turbot haematology. **Comparative Biochemistry and Physiology, Part A,** 141, 35-41.

Cal, R.M., Vidal, S., Gómez, C., Álvarez-Blázquez, B., Martínez, P. and Piferrer, F. 2005. Growth and gonadal development in diploid and triploid turbot (*Scophthalmus maximus*). **Aquaculture** (en prensa).

Cal, R.M., Vidal, S., Martínez, P., Álvarez-Blázquez, B., Gómez, C. and Piferrer, F. Growth and gonadal development of gynogenetic diploid turbot (*Scophthalmus maximus*). **Journal of Fish Biology** (en prensa).

Estos resultados han sido presentados en las siguientes comunicaciones a congresos nacionales e internacionales:

Piferrer, F., **Cal, R.M**., Álvarez-Blázquez, B., Sánchez, L., Martínez, P. 1997. Induction of triploidy in the turbot (*Scophthalmus maximus* L.) by cold shocks applied shortly after fertilization. **Abstracts** of the **Sixth International Symposium on Genetics in Aquaculture. Institute of Aquaculture University of Stirling. Escocia.** 24-29 de Junio 1997**.**

Lustres, L., Vidal, S., **Cal**, **R.M.**, Fernández-Casal, J. y Martines, P. 1999. Análisis comparado de las anomalías del esperma en rodaballo (*Scophthalmus maximus* L.), coruxo (*Scophthalmus rhombus* L.) y solla (*Pleuronectes platessa* L.). **Actas del Vll Congreso Nacional de Acuicultura. Las Palmas de Gran Canaria, España.** 18-21 de mayo 1999. pp. 172.

Piferrer F., **Cal**., **R.**, Sánchez, L. y Martínez, P. 1999. Inducción de la triploidía en el rodaballo (*Scophthalmus maximus* L). Determinación del nivel de ploidía y de los efectos de choques fríos. Producción en masa de rodaballos triploides**. Actas del Vll Congreso Nacional de Acuicultura. Las Palmas de Gran Canaria, España.**. 18-21 de mayo. pp. 178.

Terrones J, **Cal**., **R.**, Vidal, S., Gómez, C., Martínez, P. y Piferrer, F. 2001. Crecimiento y desarrollo gonadal de rodaballos (*Scophthalmus maximus* L.) triploides hasta los 18 meses de edad. **Actas del VIII Congreso Nacional de Acuicultura. Santander, España.** 22-25 de mayo 2001. pp. 245-247.

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Cuñado N., Terrones, J., Piferrer, F., **Cal**, **R.**, Sánchez, L., Martínez, P. y Santos, J.L. 2001. Synapsis in diploid and triploid males and females of turbot, *Scophthalmus maximus* (Pisces, Scophthalmidae). **Abstracts 14 International Chromosome Conference. Würzburg, Alemania.** 4-8 sept. 2001.

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Terrones, J., Vidal, S., **Cal**, **R.**, Martínez, P. and Piferrer, F. 2003. Apoptosis during gametogenesis in diploid and triploid turbot (*Scophthalmus maximus* L.). **Proceedings of the 7th International Symposium on Reproductive Physiology of Fish. Mic, Japón.**

Cal, R.M., Vidal, S., Martínez, P., Gómez, C., Álvarez-Blázquez, B. and Piferrer, F. 2004. Comparison of growth in diploid vs triploid turbot (*Scophthslmus maximus* L.) until an age of 4 years. **Europ. Aquacult. Soc. Spec. Publ., 34: 196-197.**

Fortes, G.G., Nonnis-Marzano, F., Gandolfi, G., **Cal**, **R.M.**, Piferrer, F., Bouza, C., Martínez, P. and Sánchez, L. 2004. Preliminary linkage map in turbot (*Scophthalmus maximus*) with AFLPs and microsatellite markers. **Europ. Aquacult. Soc. Spec. Publ., 34: 346-347.**

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ÍNDICE

CAPÍTULO I
INTRODUCCIÓN GENERAL

1. EL SEXO EN LOS PECES

La expresión del sexo en peces es muy variada, implicando varios tipos de cromosomas sexuales y varios tipos de reproducción, así como caracteres sexuales específicos y diversos comportamientos asociados al sexo (Piferrer, 2001; Devlin y Nagahama, 2002). La naturaleza básica de esta diversidad se puede considerar desde el mecanismo dual de la expresión del sexo: la determinación sexual que define el sexo genético, y la diferenciación sexual, que define el sexo fisiológico (Yamazaki, 1983), ambos procesos consecutivos en el tiempo (Fig. 1).

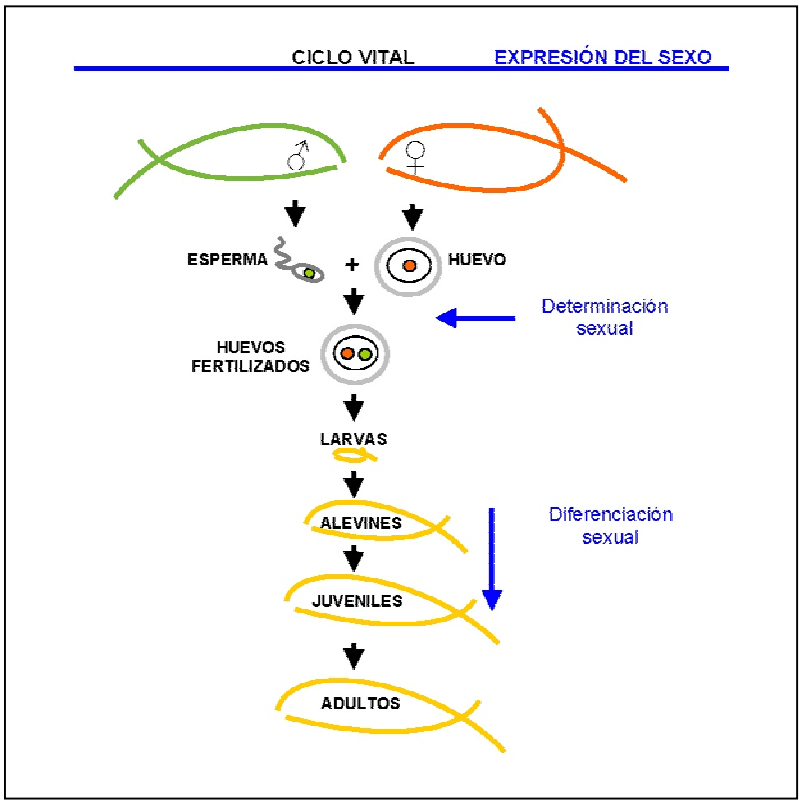

Fig. 1. Expresión del sexo en relación con las etapas del ciclo vital.

1.1. El sexo genético

Se determina en el momento de la fertilización, y es el resultado de la combinación de los cromosomas del huevo y del esperma. La determinación sexual se basa en un conjunto de genes (los genes responsables de la determinación sexual), responsables de la existencia de las gónadas, así como de su forma y de sí se diferencian en testículos u ovarios (Piferrer, 2001). Estos genes pueden estar concentrados en un par de cromosomas, llamados cromosomas sexuales, o distribuidos a lo largo del genoma

(sistema poligénico) (Bull, 1983; Tave, 1993). El sexo genético de cada individuo dependerá del conjunto de genes de determinación sexual heredado de sus padres (Piferrer, 2001).

Cuando los genes están concentrados en los cromosomas sexuales, pueden tener diferentes mecanismos de determinación sexual. Han sido propuestos 8 sistemas de determinación sexual basados principalmente en análisis citogénico y resultados de cruzamientos controlados. Estos sistemas van desde los dos más sencillos XX / XY o WZ / ZZ, los más comunes en peces, a otros seis más complejos, implicando a más de un par de cromosomas sexuales o diferente número de cromosomas dependiendo del sexo (Tave, 1993). El sexo en el que los cromosomas sexuales son iguales se llama homogamético, y en el que son distintos heterogamético. La mayoría de las especies de teleósteos de interés comercial producen machos y hembras en proporción 1:1, lo que sugiere que poseen mecanismos de determinación sexual tipo XX / XY o ZW / ZZ.

El sistema poligénico o polifactorial, es un sistema de determinación sexual donde los genes determinantes del sexo están presentes en otros cromosomas (autosomas) de la misma forma que en los heterocromosomas (Hunter y Donaldson, 1983; Price, 1984; Chourrout, 1988). Por tanto el sexo de los descendientes será el resultado de la combinación de los factores presentes en el conjunto de cromosomas heredados de cada progenitor. Los peces con sistemas de determinación sexual poligénico están caracterizados por la proporción de sexos diferentes de 1 macho:1 hembra en el resultado de un cruzamiento en concreto, típica de especies con sistema de determinación cromosómico puro. Además, las proporciones de sexos pueden ser diferentes en sucesivos lotes originados de los mismos reproductores.

1.2. El sexo fisiológico

Se desarrolla después del sexo genético, a través de procesos bioquímicos durante la ontogénesis y bajo el control del sexo genético. El sexo fisiológico incluye: el sexo gonadal, el sexo externo y el sexo etológico (Yamazaki, 1983).

El sexo gonadal se refiere al tipo de órgano sexual. La base de la fisiología sexual depende sobre todo del tipo de órgano sexual primario, y se clasifica en dos grupos, los gonocoristas y los hermafroditas. *En los gonocoristas*, cada pez tiene o testículos u ovarios, y pueden ser *indiferenciados* cuando en principio la gónada indiferenciada se desarrolla como ovario y luego la mitad aproximadamente se vuelven

machos y la otra mitad siguen como hembras, o *diferenciadas* cuando la gónada indiferenciada se diferencia directa y definitivamente en ovario o testículo.

En las especies *hermafroditas*, la mayoría de sus individuos desarrollan ovarios y testículos al mismo tiempo, con funciones de macho y hembra durante su vida. Hay tres tipos de hermafroditismo: *sincronizado* en donde los testículos y los ovarios maduran al mismo tiempo, *protogíneos* con función primero de hembras y más tarde como machos, y *proterándicos* en donde los animales empiezan funcionando como machos y más tarde los ovarios remplazan a los testículos (Atz, 1964; Ghiselin, 1969; Yamamoto, 1969; Chan, 1970).

El sexo externo se refiere a los accesorios sexuales tales como la vesícula seminal, los conductos de huevos u, otros caracteres secundarios que dan la identidad sexual externa, mientras que el sexo etológico se refiere al comportamiento sexual (Yamazaki, 1983).

En los mamíferos el sexo genético coincide con el fenotípico pero en los peces gonocoristas esto no es siempre cierto ya que en algunos casos la diferenciación sexual es alterada por factores ambientales resultando un pez con un sexo genético diferente del sexo fenotípico (Yamamoto, 1999). Parece que los efectos ambientales actúan sobre el fenotipo interviniendo en el proceso de diferenciación sexual, promoviendo o inhibiendo la expresión de ciertos genes (Piferrer, 2001).

2. MEJORA GENÉTICA DE PECES EN ACUICULTURA

En la naturaleza, la variabilidad genética en organismos que se reproducen sexualmente se origina o bien por recombinación, por mutación en el marco de la selección natural, siguiendo un plan no prediseñado. Por el contrario, la mejora genética ejercida de forma artificial se hace según un plan prediseñado, domesticando la especie, y haciendo que se reproduzcan según parámetros de capacidad de producción mediante métodos realizados a través del control y/o la manipulación de la reproducción (Colombo, 2001). Ambos tipos de métodos, se integran en programas de mejora genética desarrollados en la acuicultura, como estrategias dirigidas a incrementar la eficiencia y promover la diversificación (Gjerde y Rye, 1998).

El rápido incremento de la producción de la acuicultura en el mundo, con la consiguiente caída de los precios, ha hecho necesario hacer un esfuerzo en incrementar

la eficiencia de la producción y en la diversificación de los productos, con el fin de mantener su rentabilidad (Bartley, 1998).

Las técnicas genéticas pueden contribuir a mejorar la producción. La mejora sostenida de la producción depende en gran medida de la utilización de los recursos genéticos presentes en las poblaciones naturales y de la utilización de la tecnología adecuada para incorporar dichas variantes al cultivo (Allendorf et al., 1987; Tave, 1993). La aplicación de los principios de la genética en acuicultura es un hecho relativamente reciente y todavía hoy muchas de las especies que se cultivan son iguales o muy similares a las del medio natural, sobre todo en lo que a especies marinas se refiere. Las primeras investigaciones se empezaron a realizar durante la década de los años 60, iniciándose una serie de experimentos aplicando las técnicas de reproducción selectiva en carpa común y en salmónidos. A partir de los años 70, las investigaciones en mejora genética se empezaron a desarrollar a través de programas de cruzamiento, aplicando técnicas de mejora genética tradicional como la selección fenotípica, el cruzamiento entre líneas y razas y la hibridación interespecífica. La mejora y optimización de tales programas ha sido la base para el desarrollo de otros nuevos, y en la última década ya son numerosos los programas que se aplican a muchas especies de peces en un gran número de países (Tabla 1) (Hulata, 2001).

A finales de los años 80 y durante los 90 las nuevas tecnologías empezaron a tener impacto en acuicultura buscando mejorar las tasas de crecimiento y paliar los efectos de maduración sexual. El desarrollo de las técnicas moleculares en adición a los métodos tradicionales, ha permitido caracterizar genéticamente a los peces, determinando las relaciones de parentesco entre familias y así orientar los cruzamientos, no sólo en virtud de sus caracteres externos, sino también con el fin de evitar problemas generados por la consanguinidad.

Durante los últimos 20 años, el uso de técnicas de mejora genética como la manipulación cromosómica, la transgénesis, el mapeo genómico y el uso de marcadores de DNA han completado su fase experimental y actualmente están siendo implementadas comercialmente, para mejorar caracteres como el aspecto, la tasa de crecimiento, la eficiencia en conversión del alimento, resistencia a enfermedades, porcentaje de fertilidad y tolerancia a baja calidad del agua (Hulata, 2001). La manipulación cromosómica y/o hormonal permite la feminización, la masculinización y la esterilización en peces, para mejorar la rentabilidad del cultivo (Felip, 2000a).

Tabla 1. Especies de acuicultura para las cuales se han aplicado diferentes tecnologías de manipulación genética (Tomado de Hulata, 2001)

Tecnología	Especies implicadas en programas de cría	Especies implicadas en acuicultura práctica
Selección	Salmón Atlántico, *Salmo salar* (Chile, Islandia, Irlanda, Noruega, Escocia); salmón ´coho´, *Oncorhynchus kisutch* (Chile, EE.UU.); trucha arco iris, *Oncorhynchus mykiss* (Chile, Francia, Noruega, EE.UU.); trucha común, *Salmo trutta* (Francia); pez gato punteado, *Ictalurus punctatus* (EE.UU.); dorada, *Sparus auratus*, y lubina, *Dicentrarchus labrax* (Israel); rodaballo, *Scophthalmus maximus* (España, Noruega); carpa asiática, *Labeo rohita* (India); tilapia del Nilo, *Oreochromis niloticus* (China, Egipto, Indonesia, Filipinas, Tailandia); *Oreochromis shiranus* (Malawi); carpa común, *Cyprinus carpio* (China, República Checa, Estonia, Indonesia, Rusia, Vietnam); *Megalobrama amblycephala* (China); carpín, *Carassius carassius* (China); tenca, *Tinca tinca* (República Checa); ostra japonesa, *Crassostrea gigas* (Australia, EE.UU.); ostra de Sydney, *Saccostrea glomerata* (Australia); camarón blanco, *Litopenaeus vannamei* (Brasil, Colombia, México, EE.UU.); langostino tigre, *Penaeus monodon* (Australia, Tailandia, EE.UU.); langostino tigre japonés, *Penaeus japonicus* (Australia)	Salmón Atlántico, *Salmo salar* (Chile, Islandia, Irlanda, Noruega, Escocia); salmón del Pacífico, *Oncorhynchus* sp. (Canadá); salmón ´coho´, *Oncorhynchus kisutch* (Chile, EE.UU.); trucha arco iris, *Oncorhynchus mykiss* (Chile, Francia, Noruega, EE.UU.); dorada, *Sparus auratus*, y lubina, *Dicentrarchus labrax* (Israel); carpa común, *Cyprinus carpio* (China, República Checa, Estonia, Indonesia, Rusia, Vietnam); *Megalobrama amblycephala* (China); carpín, *Carassius carassius* (China); tenca, *Tinca tinca* (República Checa); tilapia del Nilo, *Oreochromis niloticus* (China, Egipto, Indonesia, Malasia, Filipinas, Vietnam); ostra japonesa, *Crassostrea gigas* (EE.UU.); pez gato punteado, *Ictalurus punctatus* (EE.UU.) ; camarón blanco, *Litopenaeus vannamei* (EE.UU.); camarón azul, *Penaeus stylirostris* (México); langostino tigre japonés, *Penaeus japonicus* , langostino tigre marrón, *P. esculentus* y langostino banana, *P. merguiensis* (Australia)
Cruzamientos intraespecíficos	Dorada (Israel); trucha arco iris (Irlanda); pez gato punteado (EE.UU.); carpa común (China, República Checa, Hungría); tilapia del Nilo (Malasia); ostra japonesa (EE.UU.)	Carpa común (China, República Checa, Estonia, Hungría, Israel, Rusia, Tailandia, Vietnam); tilapia del Nilo (Malasia); pez gato punteado (EE.UU.); dorada (Israel); trucha arco iris (Irlanda); ostra japonesa (EE.UU.)
Hibridación	Trucha arco iris x *Oncorhynchus clarkii* (Francia); pez gato punteado y azul (EE.UU.); pez gato asiático y africano, *Clarias macrocephalus* x *C. gariepinus* (Vietnam)	Tilapia (China, Israel, Taiwan); pez gato punteado y azul (EE.UU.); pez gato asiático y africano, *Clarias macrocephalus* x *C. gariepinus* (Vietnam); perca rayada híbrida (EE.UU., Israel, Taiwan)
Manipulación sexual	Trucha arco iris y trucha común (Francia); pez gato punteado (EE.UU.); tilapia del Nilo (Filipinas); barbo plateado (Tailandia)	Tilapia del Nilo (China, Fiji, Filipinas, Tailandia, EE.UU., Vietnam); tilapia del Jordán (Israel); salmón Atlántico (Canadá) y salmón ´coho´ (Canadá, Japón), trucha arco iris (Francia, Japón); salmón cherry, *Oncorhynchus massou* (Japón)
Ginogénesis/líneas clonales	Trucha arco iris y trucha común (Francia); salmón Atlántico, cherry, 'coho' y amago (Japón); ayu (Japón); carpa común (China, Israel, Japón); carpa dorada y carpín (Japón); barbo plateado (Vietnam); pargo japonés (Japón)	Trucha arco iris y común (Francia); carpa común (China, Japón); hirame, *Paralichthys olivaceus* (Japón)
Androgénesis	Trucha arco iris (Japón, EE.UU.); salmón amago (Japón); carpa común (Japón, Holanda)	
Poliploidía	Trucha arco iris (Francia, Japón); trucha común (Francia); ostra japonesa (EE.UU.); almeja catarina, *Argopecten ventricosus*, escalopa, *Nodipecten subnodosus*, abalón rojo, *Haliotis rufescens* (Japón)	Trucha arco iris (Canadá, Francia, Japón); ostra japonesa (Australia, EE.UU., Japón); almeja catarina, *Argopecten ventricosus* (México); salmón Atlántico (Canadá); salmón cereza, amago, ayu y hirame (Japón)
Transferencia genética	Salmón Pacífico (EE.UU., Canadá, Nueva Zelanda)	
Marcadores de ADN	Salmón Atlántico (Noruega); salmón ´coho´ (Chile)	Salmón Pacífico (Canadá)
Criopreservación de gametos/embriones	Salmón Atlántico (Canadá, Noruega, EE.UU.); salmón del Pacífico (Canadá); trucha arco iris (Noruega); carpas (China, Vietnam); ostra japonesa (EE.UU.)	Salmón Atlántico y Pacífico (Canadá)

Las técnicas genéticas no solo se utilizan para mejorar la producción sino que también se utilizan como herramienta para la conservación de la diversidad biológica acuática, desempeñando un papel importante en la conservación de los recursos. La producción de peces estériles (triploides o híbridos), y de poblaciones monosexo, minimiza la posibilidad de que peces procedentes de la acuicultura se reproduzcan entre sí y entren en competición, desplazando las especies autóctonas, o se mezclen con las poblaciones naturales afectando a su constitución genética (Hindar et al., 1991). Técnicas como la androgénesis y ginogénesis pueden reconstruir el genoma de las especies en peligro de extinción (Bartley, 1998).

Los programas de mejora genética están diseñados para conseguir la mejora a largo plazo o a corto plazo. En los programas a largo plazo, las técnicas utilizadas permiten obtener en cada generación una ganancia genética pequeña, pero que es aditiva, lo que significa que para obtener la mejora genética total que se pretende se necesitan varias generaciones. Para desarrollar programas de mejora a largo plazo es necesario disponer de un gran número de peces y hacer un seguimiento durante varios años. La técnica utilizada en programas a largo plazo es normalmente la selección.

En los programas de mejora genética a corto plazo la ganancia genética es inmediata y la mejora pretendida se consigue en una o dos generaciones, pero no es normalmente acumulativa. Para desarrollar estos programas no es necesario disponer de muchos peces pero si es imprescindible tener un lote adecuado de reproductores, productores de gametos de alta calidad. Las técnicas utilizadas en los programas a corto plazo son los cruzamientos intraespecíficos, los cruzamientos interespecíficos (llamados también hibridación), la manipulación cromosómica (poliploidización, la ginogénesis, la androgénesis) y la manipulación fisiológica del sexo (control hormonal).

2.1. Técnicas de mejora genética clásica a través del control de los apareamientos

Estas técnicas de mejora genética se ejercen directamente sobre los peces adultos, y se aplican mediante el control de los apareamientos. Se basan en decidir y controlar los individuos que participan en cada cruzamiento. Estas técnicas son: la selección fenotípica, el cruzamiento entre líneas y la hibridación interespecífica, las dos primeras son las más utilizadas combinándose frecuentemente en los programas de mejora genética.

2.1.1. Selección

La selección es una técnica que se realiza eligiendo de acuerdo al fenotipo los peces que presentan el carácter o la combinación de carácteres que más interesan, y éstos se utilizan como reproductores para originar la siguiente generación. Al diseñar el protocolo es necesario determinar el carácter que interesa y el parámetro de evaluación del resultado de selección, y constatar que el carácter elegido sea hereditario y no sea debido a condiciones ambientales de cultivo.

Entre los carácteres que se consideran a mejorar el más frecuente es el incremento de la tasa de crecimiento (Tave, 1986), aunque también hay otros interesantes como la reducción de los intervalos de generación, la resistencia a enfermedades o a determinados agentes polucionantes característicos de una determinada región (Lourdes et al., 1995). En la aplicación de esta técnica es necesario evitar cambios en carácteres no deseados y la endogamia que se puede producir cuando se utiliza un número reducido de reproductores (Gjerde y Rye, 1998).

La selección se puede hacer sobre individuos, sobre familias o combinada, eligiendo a los mejores individuos dentro de familias seleccionadas (Wohlfarth, 1990; Bartley, 1998). Este método es lento, sobre todo en especies de ciclo de vida largo, y además se requiere mantener a un gran número de reproductores.

Hoy en día la incorporación de marcadores de DNA a los programas de selección (selección asistida) ha mejorado su eficiencia. Los programas de selección incorporando marcadores moleculares de DNA como, por ejemplo, los microsatélites para determinados carácteres, permite mejorar las características que pueden ser hereditarias tales como tasa de crecimiento, la fecundidad, o la resistencia a enfermedades. La selección asistida por marcadores de DNA es una práctica fácil de realizar a pequeña escala comercial en las granjas, ya que no se necesitan especiales condiciones que alteren la rutina del cultivo, y los peces no necesitan ser marcados. Los análisis genéticos se pueden realizar en fragmentos de aleta o biopsias que no alteran su aspecto para la futura comercialización (Herbinger et al., 1995).

2.1.2. Hibridación

La hibridación es otro método tradicional que se basa en el cruzamiento de dos grupos genéticamente diferentes para combinar cualidades de los dos grupos

(Wohllfarth, 1990; Colombo et al., 1998). El resultado de la hibridación es imprevisible, ya que depende de la combinación de los alelos (Tave, 2003).

La hibridación puede ser intraespecífica, cuando los cruzamientos se hacen entre líneas o razas distintas de la misma especie, o interespecífica cuando se cruzan animales de especies distintas. Normalmente la primera generación de descendientes es la que interesa a los acuicultores. Los híbridos pueden ser fértiles o estériles, y en las siguientes generaciones los cruzamientos pueden ser entre ellos, o con individuos de las líneas parentales, o con otros individuos, aunque es muy importante mantener las líneas parentales, ya que en muchos casos los híbridos son estériles.

Esta técnica es muy fácil de utilizar y el resultado se observa a corto plazo, pero es un resultado limitado a esta generación, por lo que cuando un considerable componente de la varianza genética en la población es no aditiva en el programa de reproducción deben combinarse la hibridación con la selección (Gjedrem, 1985). En general, la hibridación es utilizada para conseguir peces mejores, mientras que la selección se utiliza para conseguir lotes de reproductores mejores.

La hibridación interespecífica aún presenta muchas incógnitas respecto al potencial reproductivo de muchos híbridos así como a la viabilidad de estos en la naturaleza (Colombo et al., 1998).

2.2. Técnicas de mejora genética a través de la manipulación de la reproducción

La mejora genética ejercida a través de la manipulación de la reproducción se basa en la aplicación de una serie de técnicas de manipulación cromosómica y/o manipulación fisiológica del sexo, que se ejercen sobre los gametos, los embriones, las larvas o los alevines.

2.2.1. Manipulación cromosómica

La base del control del sexo genético o manipulación cromosómica radica en la posibilidad de manipular el conjunto de genes heredado de los padres, decidiendo qué cromosomas irán en el cigoto (Thorgaard, 1983). Las técnicas de manipulación cromosómica consisten en la manipulación de juegos enteros de cromosomas alterando las divisiones celulares o inactivando el DNA de los gametos. Las más utilizadas son la inducción de la poliploidía (triploidía y tetraploidía), de la ginogénesis

(meioginogénesis y mitoginogénesis) y de la androgénesis. Estas técnicas se aplican en el momento de la fertilización e inciden sobre el proceso de determinación sexual (Fig. 2).

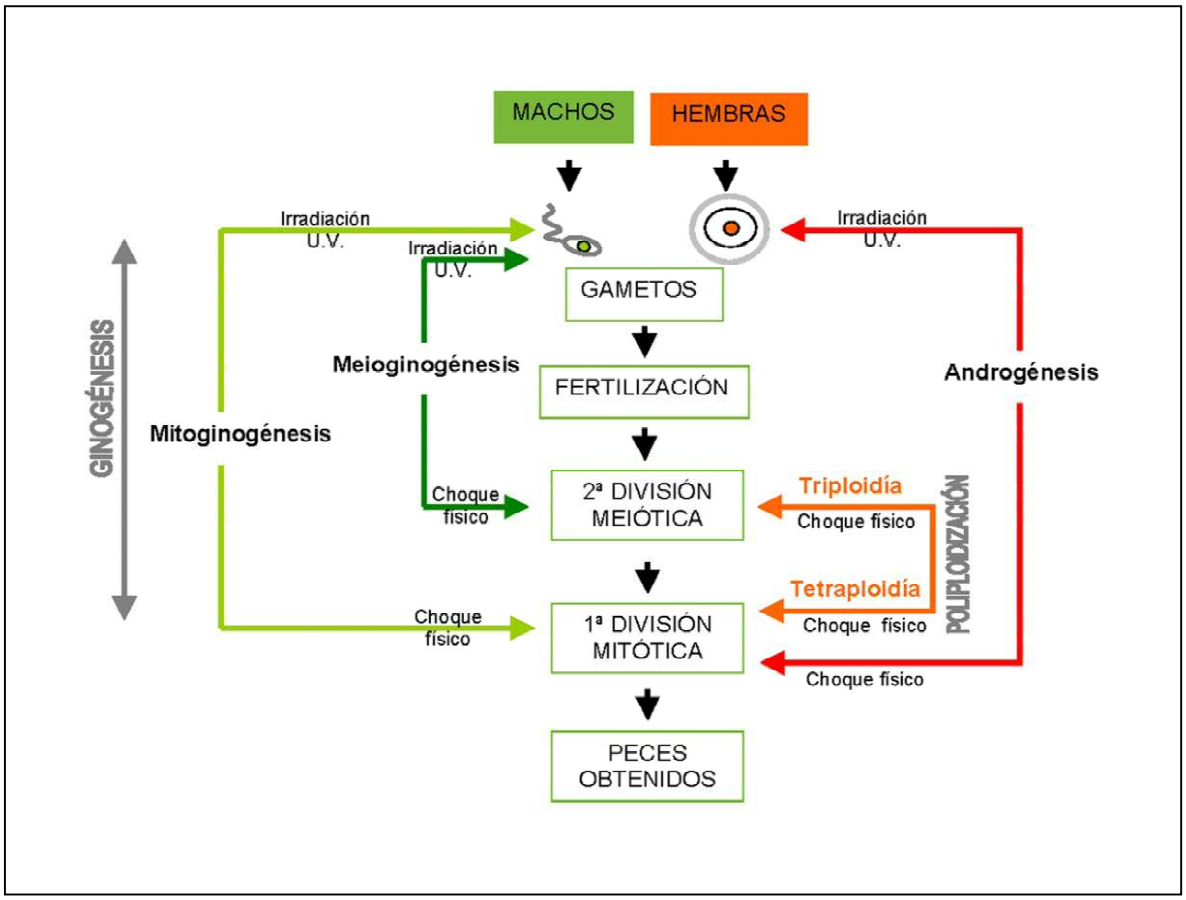

Fig. 2. Manipulación cromosómica en peces en relación a la etapa de su ciclo vital.

A) La triploidía

Por organismo poliploide se entiende aquel que posee más de un número 2n de cromosomas en sus células somáticas. Así los triploides son individuos poliploides con tres conjuntos de cromosomas, los tetraploides con cuatro y los hexaploides con seis (Dunham, 2004). La poliploidía más utilizada en acuicultura es la triploidía.

La triploidía es inviable en mamíferos y aves pero es viable en peces (Chourrout et al., 1986). En el medio natural la mayoría de las especies de peces teleósteos tienen un numero diploide de cromosomas, pero la triploidía puede aparecer de forma natural en individuos no tratados (Dunham et al., 2004; Le Comber y Smith., 2004), y ya ha sido documentada en salmón rosado (Utter et al., 1983), y en especies de familias como Poeciliidae (Cimino, 1972), Cyprinidae (Gold y Avise, 1976) y Salmonidae (Thorgaard

y Gall, 1979). De hecho el 63% de las especies de peces pertenecen a 9 órdenes en los cuales se incluyen poliploides. La poliploidía afecta a todos los niveles del genoma y puede influir el tipo de cambios evolutivos observados o simplemente las tasas a los que ellos ocurren (Le Comber y Smith., 2004). Finalmente hay especies en que coexisten diploides y tetraploides (Saitoh et al., 1984).

Inducción a la triploidía

La inducción artificial de la triploidía tiene como objeto la producción de individuos con un conjunto extra de cromosomas. Se empezó a desarrollar en principio para especies de agua dulce (Swarup, 1959), pero en los últimos veinte años, ha habido un gran desarrollo en la investigación y aplicación de esta técnica en especies marinas de las familias Pleuronectidae, Sparidae, Paralichthyidae, Moronidae, Soleidae y Scophthalmidae (Felip et al., 2001a).

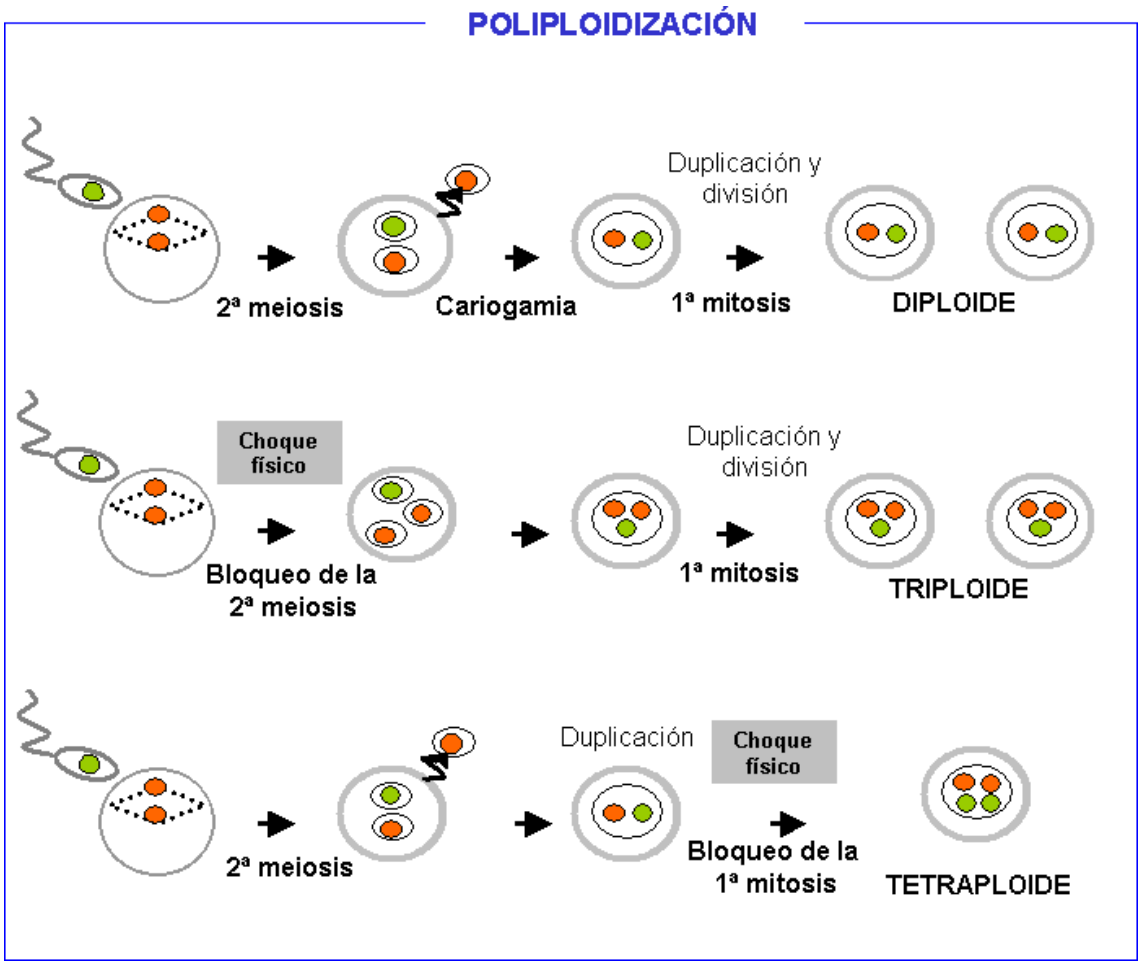

Fig. 3. Inducción a la triploidía (Colombo, 2001). La triploidía se obtiene impidiendo la extrusión del segundo corpúsculo polar justo después de la fertilización aplicando un choque físico. La tetraploidía se obtiene permitiendo la división meiótica pero bloqueando la primera división mitótica del cigoto.

Los métodos de inducción de la triploidía se basan en la alteración de las divisiones celulares, de la segunda meiosis (métodos directos), o de la primera mitosis (métodos indirectos) (Fig. 3). La meiosis es el fenómeno biológico de mayor importancia en los organismos de reproducción sexual, y es la base de las técnicas para manipular el genoma de las especies acuáticas. Este proceso genera la diferencia del número de cromosomas presentes en las células somáticas vs los gametos, produciendo gametos con la mitad de los cromosomas que las células somáticas. Implica dos divisiones sucesivas tras las cuales las células germinales diploides con 2n cromosomas, pasan a cuatro células haploides con n cromosomas (Tave, 1993; Alberts et al., 1994). En el proceso meiótico de las hembras en los teleósteos, la primera división meiótica de los oocitos de primer orden se inicia después de la eclosión Estos oocitos completan la división meiótica I, formando un pequeño corpúsculo y un oocito de segundo orden, y entran en la metafase de la división meiótica II. Aquí se detiene y finaliza meses más tarde con la ovulación de oocitos secundarios después, cuando es estimulado por la fecundación y empieza el desarrollo embrionario. Así, la segunda división meiótica se completa solamente después de la fertilización (Carrasco, 1998).

La mitosis a diferencia de la meiosis, produce células hijas con el mismo complemento genómico que la célula de la que se originan, y cada cromosoma derivado del padre o de la madre se comporta de manera autónoma e independiente.

Los *métodos de inducción* son *directos,* cuando los tratamientos se aplican a las células que se convertirán en individuos triploides, se realizan normalmente minutos después de la fertilización y provoca la interrupción de la segunda división meiótica impidiendo la extrusión del segundo corpúsculo polar (Tiwary et al., 2004). El resultado es un cigoto triploide que contiene el genoma presente en el óvulo, el genoma presente en el espermatozoide y el genoma presente en el segundo corpúsculo polar (Carrasco, 1998).

En los *métodos de inducción indirectos*, los tratamientos se aplican sobre células que darán lugar a individuos tetraploides. Se aplican más tarde que los directos, incluso después de varias horas de haberse producido la fertilización, e inhiben la primera división mitótica del cigoto. El resultado es un cigoto en el que el ADN ha sido replicado y que contiene un número diploide de cromosomas. Los choques tardíos producen individuos tetraploides con gametos diploides. Posteriormente al fecundar un

huevo normal con esperma 2n, de un individuo tetraploide, se producirán individuos 3n. Esta generación de tetraploides podrá ser utilizada a continuación en diferentes tipos de cruces diseñados para producir los individuos triploides, evitando tener que utilizar los métodos directos cada vez que se requieren individuos triploides (Thorgaard, 1983).

Estos métodos se utilizan cuando se quiere producir un gran número de individuos y se basan en el supuesto de que individuos tetraploides producen gametos diploides. También se utilizan en especies en los que el uso de los métodos directos es complicado, por ejemplo por la dificultad de la recolección de gametos. Pero la viabilidad de estos individuos es limitada, así como su baja fertilidad, lo que hacen que esta alternativa no tenga mucho futuro (Carrasco 1998).

Los tratamientos pueden ser de tres tipos:

Los choques térmicos, de calor o de frío, han sido utilizados con éxito en la producción de individuos triploides en muchas especies acuáticas. Se trata de aplicar cambios bruscos de la temperatura a huevos poco tiempo después de la fertilización. La temperatura, la duración y el momento de aplicación de estos tratamientos varían dependiendo de cada especie. Los choques térmicos, son los más utilizados por la industria debido a su simplicidad, su bajo coste y su eficiencia. En los choques térmicos, no sólo hay que considerar la temperatura del choque en si, sino la diferencia de temperatura antes y durante el choque (Ihssen et al 1990).

Los *choques de presión* presentan la ventaja de aplicar un tratamiento más uniforme a los huevos, solucionando las dificultades de la transferencia uniforme de temperatura en los choques térmicos, especialmente en especies en las que los huevos son de gran tamaño. La duración de los choques de presión es más corta que la de los choques térmicos, lo que puede presuponer una reducción de la mortalidad que no siempre se da, aunque también han sido descritas anormalidades en el desarrollo embrionario y aberraciones cromosómicas tras su aplicación (Carrasco, 1998).

Las *sustancias químicas* más utilizadas han sido los alcaloides como la colchicina, la citocalasina B, y sustancias con acción anestésica como el oxido nitroso, pero son altamente inductores de formación de tumores y muy citotóxicos, por lo que su uso se limita a la investigación (Johnstone, 1985; Felip, 2000a).

Evaluación de la ploidía

Las técnicas para la producción de individuos triploides no siempre son totalmente efectivas y por eso la ploidía debe ser confirmada en una muestra significativa o en todos los individuos nacidos de huevos tratados para eliminar la posibilidad de que algunos individuos maduren, interfiriendo con los sistemas de producción. Para que resulten rentables en instalaciones comerciales, los métodos utilizados deben poder analizar un gran número de individuos y, además, ser lo más rápidos y baratos posible. Estos métodos se clasifican en tres tipos, citogenéticos, citológicos y de cuantificación de ADN nuclear (Carrasco, 1998).

Los *métodos citogenéticos* se basan en la determinación del número de los cromosomas o de las regiones organizadoras de los nucleolos. La producción del cariotipo es un método barato y no requiere grandes equipos, y sigue siendo el único método para determinar con precisión el número y morfología de los cromosomas, pero es laborioso y no es practico cuando se trata de identificar gran número de individuos. Para contar los cromosomas se hace sobre placas metafásicas o sobre el cariotipo, que es la presentación de los cromosomas de un individuo (generalmente en metafase) en longitud decreciente y agrupando cromosomas homólogos en base a su morfología y posición del centrómero. Los mejores cariotipos se obtienen de preparaciones de embriones o peces muy jóvenes (Carrasco, 1998).

El número de regiones organizadoras del nucleolo (NOR) teñidas con nitrato de plata es también un método fiable y sencillo, pero se necesita conocer las características citogenéticas de la especie a analizar para poder deducir el nivel de ploidía a partir del número de regiones organizadoras del nucleolo en el cariotipo (Carrasco 1998).

Los *métodos citológicos* se basan en la determinación del tamaño celular o nuclear, normalmente en eritrocitos. El aumento en la cantidad de ADN y cromosomas que causa la poliploidización se traduce en un aumento en el tamaño nuclear y celular (Carrasco, 1988). La medición microscópica de las dimensiones nucleares o celulares es una técnica relativamente barata y sencilla que requiere únicamente un microscopio, y es la más útil cuando el tamaño de los peces es suficiente para poder extraerles sangre sin recurrir al sacrificio. Presenta el inconveniente del consumo de tiempo en la extracción de sangre, elaboración y tinción del frotis, y el análisis cuando se trata de gran número de peces (Carrasco, 1998).

La posibilidad de automatizar el proceso de medición electrónica del volumen nuclear o celular de los eritrocitos con un contador de partículas resulta altamente favorable (Tiwary et al., 2004). El contador detecta la diferencia de tamaño de la partícula y así provee de un método rápido y eficiente para la estimación de la ploidía. Pero los costes de su utilización son altos, lo que hace que esta técnica solo sea rentable en explotaciones de gran tamaño.

Los *métodos de cuantificación del ADN* se basan en la cuantificación fluorimétrica o colorimétrica del ADN nuclear mediante tinciones o pigmentos con afinidad especifica por el ácido desoxiribonucleico (DAPI, etc). La citometría de flujo puede ser utilizada para analizar con gran rapidez el contenido de ADN de un alto número de células en interfase, con una precisión mayor que la alcanzada por cualquier otra técnica cuantitativa. Pero el elevado coste del instrumento, así como la formación especializada que se requiere hace que este método de determinación sea probablemente de difícil aplicación en explotaciones acuícolas.

Efectos de la triploidía

El aumento de tamaño del núcleo debido a la necesidad de alojar un conjunto más de cromosomas y el que en consecuencia se produce en el citoplasma para mantener la tasa núcleo/citoplasma constante, resulta en un aumento del tamaño de las células somáticas en la mayoría de los tejidos de los peces triploides (Swarup, 1959).

En teoría, el aumento de tamaño celular debería dar lugar a que los peces triploides fueran más grandes que los diploides, pero esto no es siempre cierto, y generalmente la condición de triploides en peces no modifica su normal apariencia externa. Así en algunas especies los triploides crecen más (Purdom, 1976; Thorgaard and Gall, 1979; Wolters et al., 1981), en otras crecen lo mismo (Don y Avtalion 1986; Dunham, 1990; Hussain et al., 1995) y en otras crecen menos (Chourrout et al., 1986) que los diploides. Que los triploides no crezcan más que los diploides puede ser debido a una disminución observada en el número de células en los tejidos y órganos que contienen estas células más grandes, como resultado de una reducción en la tasa de mitosis (Dunham, 2004).

El desarrollo embrionario normalmente es similar entre ambas ploidías. Quillet et al. (1988) describen en la trucha arco iris un ligero retraso en el desarrollo embrionario desde la fertilización hasta la eclosión, mientras que Gray et al. (1993) no

encuentran diferencias consistentes en el desarrollo embrionario en seis especies de salmónidos.

La triploidía induce esterilidad. El desarrollo gonadal es interrumpido en la gametogénesis cuando las células entran en meiosis debido a la imposibilidad del apareamiento entre cromosomas homólogos, ante la existencia de un tercer conjunto de cromosomas (Gui et al., 1992; Cuñado et al., 2001; 2002). Suele tener distinta incidencia en machos y en hembras. En las hembras, los ovarios son reducidos y de aspecto rudimentario. La esterilidad puede ser debida a los bajos niveles de gonadotropina y esteroides sexuales durante la fase de vitelogénesis del ciclo reproductivo (Tiwary et al., 2004). En los machos, los testículos se desarrollan casi en la misma forma que los diploides y en ocasiones llegan a producir espermatozoides, pero en número reducido y presentando muchas veces anormalidades (Nakamura et al., 1993). Los espermatozoides de peces triploides son generalmente aneuploides (Allen et al., 1986; Benfey et al., 1986), y cuando fecundan huevos diploides los embriones resultantes son también aneuploides (Ueda et al., 1987) y mueren durante el desarrollo embriónico. Los machos de algunas especies, como el salmón Atlántico, pueden incluso desarrollar caracteres sexuales secundarios, pero sus gónadas son pequeñas y presentan anormalidades.

Generalmente durante la etapa juvenil los triploides no suelen crecer más que los diploides, pero a partir de la maduración sexual es frecuente que los triploides, debido a su esterilidad, crezcan más rápido que los diploides, que tienden a ralentizar el crecimiento o incluso detenerlo durante los periodos de maduración. No obstante, en algunas especies, los diploides exhiben un crecimiento compensatorio tras las puestas, lo que puede eliminar la ventaja adquirida por los triploides. Los triploides tienen similares características merísticas que los diploides, aunque varias anormalidades han sido asociadas con la triploidía siendo probablemente la más frecuentemente descrita el desarrollo de deformidades en la mandíbula (Sutterlin et al., 1987).

Los peces triploides toleran mal los bajos niveles de oxígeno. En las células de la sangre, el aumento de volumen altera la relación superficie/ volumen reduciendo el área disponible y además el aumento de tamaño no es uniforme, alterándose la morfología celular (Benfey, 1999). Estas alteraciones de volumen y de forma pueden afectar el intercambio de oxígeno o de metabolitos entre el interior y el exterior de las células en

el sistema circulatorio (Ueno, 1984) y ser la causa de la menor tolerancia de los peces triploides a bajos niveles de oxígeno.

El sistema inmunitario en triploides es similar al de los diploides (Kusuda et al., 1991; Yamamoto y Lida, 1995a). La actividad de complemento permanece más alta en las hembras triploides durante el periodo de las puestas de las hembras diploides, lo que explica en parte el incremento de supervivencia en las hembras triploides en los periodos de postpuestas (Yamamoto y Lida, 1995b). Ambas ploidías responden de forma similar a la vacunación (Yamamoto y Lida, 1995a) y al stress (Biron y Benfey, 1994).

Aplicaciones de la triploidía

El objetivo principal de la inducción de la triploidía en especies acuáticas de interés comercial radica en la esterilidad que esta lleva normalmente asociada (Allen and Stanley, 1981a,b; Wolters et al., 1982; Purdon, 1983). La esterilidad en triploides se debe a un desarrollo gonadal rudimentario (esterilidad gonadal), o a la infertilidad de los gametos (esterilidad gamética) debida a las anormalidades meióticas causadas por el conjunto extra de cromosomas (Yamazaki, 1983).

El cultivo de individuos estériles es muy interesante ya que elimina los problemas asociados a la maduración sexual y puestas, como son la ralentización del crecimiento, el deterioro de la calidad de la carne y la mayor mortalidad (Bye y Lincoln, 1986; Thorgaard, 1986; Hussain et al., 1995; Dunham, 1996; Tiwary et al., 2004). En algunas especies, durante la madurez sexual el inhibido o reducido desarrollo gonadal permite que los individuos canalicen la energía no utilizada en los procesos reproductivos en crecimiento somático, alcanzando así mayor tamaño en la etapa adulta. La esterilidad inducida se aplica a las especies con desarrollo sexual precoz, y es especialmente interesante en aquellas en las que la maduración sexual empieza antes de que alcancen el tamaño comercial.

El control de la reproducción por la esterilidad, también es deseable en situaciones de superpoblación con problemas asociados de merma del crecimiento (Thorgaard y Allen, 1987) y también impide o evita el riesgo del establecimiento permanente de especies exóticas (Colombo et al., 1998).

Una ventaja adicional de la esterilidad inducida por la triploidía, es la típica mayor viabilidad de los híbridos triploides en comparación con los diploides (Chevassus

et al., 1983; Scheerer y Thorgaard, 1987). La esterilidad en híbridos hace posible la combinación de carácteres deseables de dos especies en un híbrido estéril (Parsons et al., 1986; Scheerer y Thorgaard, 1983, 1987). La triploidía además previene el cruzamiento de los híbridos con las líneas parentales (Curtis et al., 1987). Los peces triploides tienen más alta heterocigosidad que los diploides (Allendorf y Leary, 1984), lo que se puede aumentar aún más utilizando híbridos.

La triploidía tiene otras aplicaciones como prevenir la interacción entre peces cultivados escapados de jaulas y peces salvajes (Donaldson, 1996, 1997), impidiendo su cruzamiento, y puede ser usado en investigación básica en estudios de interacción entre crecimiento y reproducción, fisiología de la reproducción y funcionamiento cromosómico (Felip et al., 2001a).

La triploidía inducida presenta también algunas desventajas, como la dificultad de obtener triploidía en el 100% de los casos (Thorgaard et al., 1995), la baja tasa de crecimiento en la pubertad en algunas especies, especialmente cuando son cultivados con diploides (Carter et al., 1994; Tave, 1993) y posiblemente la menor capacidad respiratoria de los triploides debido a la desfavorable tasa de superficie/volumen de los eritrocitos de algunas especies (Aliah et al., 1991; Benfey, 1999).

B) La ginogénesis y la androgénesis

La ginogénesis y la androgénesis son dos formas de reproducción en las cuales los descendientes portan herencia solamente de un progenitor, de la madre en el caso de los ginogenéticos y del padre en el caso de los androgenéticos.

Inducción a la ginogénesis

La ginogénesis es una forma rara de reproducción sexual que produce descendientes que portan herencia exclusivamente materna. En la naturaleza es utilizada por especies cuyas poblaciones aparecen constituidas casi exclusivamente por hembras y, la reproducción se realiza con la participación de machos de especies diploides próximas. El núcleo del esperma una vez que ha penetrado en el huevo se inactiva en el citoplasma del huevo y el desarrollo del embrión es controlado únicamente por la herencia materna (Cherfas, 1981). En el medio natural ha sido descrita en la carpa *Carassius auratus (Cyprinidae)*, *y en v*arias especies de la familia Poeciliidae (Schultz, 1967; Vrijenhoek y Schultz, 1974).

En la inducción de la ginogénesis, la fertilización se realiza con el esperma heterólogo, o con esperma homólogo cuyo DNA haya sido previamente genéticamente inactivado (Luckenbach et al., 2004). En ambos casos se elimina la participación del genoma paterno en la formación del embrión, lo que motiva que la descendencia lleve herencia exclusivamente materna. Los métodos utilizados en la inactivación del esperma son:

Diferentes tipos de radiación: Ultravioleta, (UV), con rayos X y con rayos gamma (Thorgaard, 1983, 1986, 1995; Utter et al., 1983; Yamazaki 1983; Ihssen et al., 1990; Mair, 1993; Arai, 2001) pero el método más utilizado es la radiación con luz UV, que ha sido utilizado con éxito en muchas especies (Thorgaard, 1983; Felip et al., 1999).

La radiación UV tiene una capacidad de penetración muy baja, lo que hace que sólo pequeñas cantidades de esperma puedan ser tratadas, pero su manipulación es menos peligrosa que las radiaciones ionizantes. La tasa de radiación UV ha de ser definida para cada especie, determinando en cada caso la dosis de radiación donde se produce el "efecto Hertwig", aquel en el cual la inactivación del DNA del gameto es total, pero que aún conserva la capacidad para desencadenar la división celular (Felip et al., 1999). *La radiación con luz UV*, afecta la movilidad del esperma (Goudie et al. 1995; Felip et al., 1999), lo que resulta en bajas tasas de fertilización y tiene además un efecto acusado sobre el desarrollo del embrión y la eclosión en especies como *Esox masquinongy* (Lin y Dabrowski, 1996); *Oryzias latipes* (Bass y Sistrun, 1997); *Dicentrarchus labrax* (Felip et al., 1999). El esperma una vez tratado es necesario protegerlo de la luz visible, para evitar el efecto de fotoreactivación.

Otro método utilizado para inducir la ginogénesis es *el uso de sustancias químicas* con efecto mutagénico, pero estos compuestos, aunque con frecuencia menor que las radiaciones X o gamma, también producen fragmentos cromosómicos que pueden dar lugar a la persistencia de carácteres paternos en la descendencia. Los embriones y larvas haploides (si es que llegan a eclosionar), presentan muy baja viabilidad y severas malformaciones.

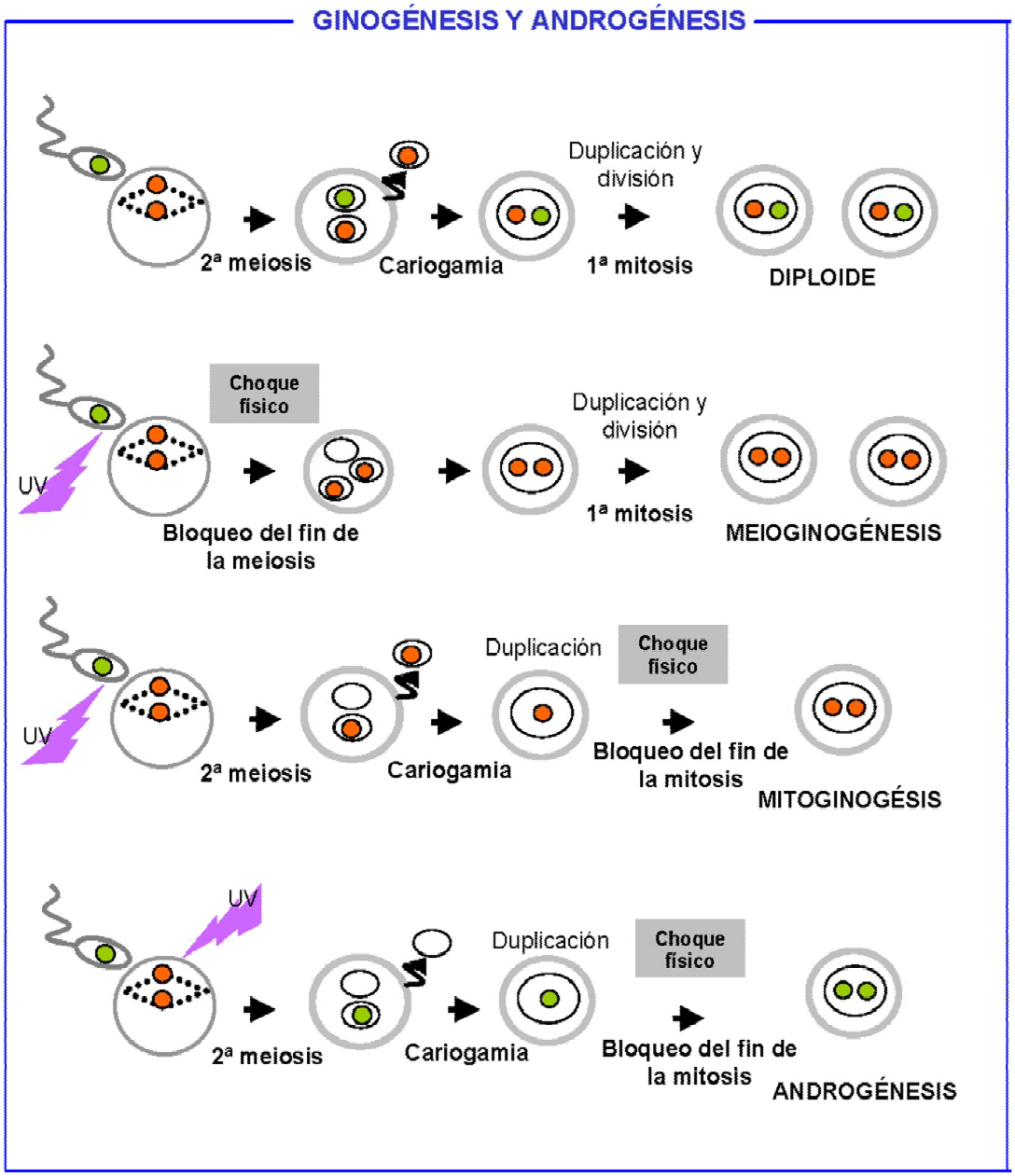

Fig. 4. Inducción a la ginogénesis y la androgénesis. La ginogénesis puede obtenerse con la activación del huevo con esperma genéticamente inactivado. El restablecimiento de la diploidía puede hacerse por el bloqueo de la segunda división meiótica del huevo (meioginogenéticos) o la primera división mitótica del cigoto (mitoginogenéticos). La herencia es en ambos casos exclusivamente materna. En la androgénesis se da la situación inversa, con la inactivación del DNA del huevo.

Los embriones resultantes de la fertilización de huevos con esperma irradiado son haploides y por lo tanto inviables, y si no se les restaura la diploidía mueren durante el desarrollo del embrión o inmediatamente a la eclosión. Tanto los embriones como las larvas recién eclosionadas, muestran severas malformaciones reflejando, además del

efecto del tratamiento, la homocigosidad engendrada. La restauración de la diploidía se consigue aplicando una de las técnicas descritas en la inducción de la triploidía. Si se actúa sobre la segunda división meiótica se producen meioginogeneticos diploides, y si se actúa sobre la primera división mitótica se producen mitoginogenéticos diploides (Fig. 4).

Los diploides meioginogenéticos conservan cierta heterocigosidad a causa de los cruzamientos durante el apareamiento entre los cromosomas homólogos en la primera meiosis, mientras que los mitoginogenéticos diploides son completamente homocigóticos en cada locus (Thorgaard, 1983; Ihssen et al., 1990). Esta homocigosidad acarrea efectos deletéreos que se traducen en una más baja viabilidad que los meioginogenéticos (Leary et al., 1985; Ihssen et al., 1990). Estos mitoginogenéticos diploides se pueden clonar, induciendo meioginogénesis en una segunda generación (Fig.5). En teleósteos marinos, la inducción de mitoginogénesis diploides ha sido descrita en lubina y dorada con muy bajo nivel de supervivencia (Colombo et al., 1996) y en trucha arco iris (Quillet, 1994).

Inducción a la androgénesis

La inducción de la androgénesis consiste en la fertilización de huevos (cuyo DNA ha sido desactivado) con esperma diploide, o con esperma haploide seguido de la aplicación de un choque de presión para restablecer la diploidía. El objetivo de la inducción de la androgénesis es conseguir individuos con herencia exclusivamente paterna.

Los métodos utilizados en la inactivación del huevo, son radiaciones que pueden ser gamma, rayos X o radiación UV. Las radiaciones gamma y las X son las más utilizadas para inactivar el núcleo del oocito, pero su empleo requiere instalaciones radioactivas especiales (Parsons and Thorgaard, 1984; Bongers et al., 1994).

La diploidía se restaura suprimiendo la primera división mitótica (Parsons and Thorgaard, 1985; Yamazaky, 1983) (Fig.2). La inhibición de la primera división mitótica conlleva el aumento de la homocigosis, y por este motivo los individuos generados tienen baja supervivencia, lo que hace que no sea rentable su producción para engorde y consumo, pero si para su utilización como reproductores en la producción de poblaciones monosexo masculinas. La androgénesis se ha utilizado en peces planos como la platija (*Platichthys flesus*) y el salmón (*Oncorhynchus masou*) y en varias

especies de truchas. También se ha utilizado con éxito radiación UV, en carpa común y tilapia del Nilo.

Evaluación de la herencia uniparental

En peces ginogenéticos o androgenéticos la condición de diploide puede ser fácilmente identificada por medio de análisis de cariotipo o de NOR, pero la demostración de su condición de pez con herencia exclusivamente materna o exclusivamente paterna solo puede ser demostrada usando marcadores morfológicos o marcadores genéticos bioquímicos o moleculares (Johnstone y Stet, 1995; Leclerc et al., 1996; Van Eenennaam et al., 1996; Peruzzi y Chatain, 2000; Felip et al., 2000b).

Los marcadores morfológicos se refieren a variantes morfológicas que puedan mostrar los peces ginogenéticos o androgenéticos, pero el diagnóstico no es concluyente y es necesario confirmarlo por otro marcador. Un marcador morfológico es la herencia de patrón de color ligado al sexo. Así, por ejemplo, si en una especie los machos y las hembras tienen un patrón de color distinto, los individuos ginogenéticos deberían tener sólo el patrón correspondiente a las hembras. Las larvas y embriones haploides muestran alteraciones morfológicas denominadas en su conjunto como "el síndrome de la haploidía", y aunque esto es característico embriones que contienen un solo conjunto de cromosomas, no excluye que puedan ser debido a otras causas, y solo sirve para la identificación de posibles ginogenéticos (Felip et al., 2000b).

Los marcadores genéticos basados en estudios de proteínas y/o aloenzimas tienen pocas aplicaciones ya que tienen dos limitaciones que son la baja proporción de loci polimórficos y la reducida diversidad alélica de estos loci (Magoulas, 1998).

Los marcadores de DNA nuclear son los mas recientemente introducidos en acuicultura y entre ellos los microsatélites son los más utilizados. Estos marcadores de ADN están constituidos por un motivo corto de repetición organizado en tándem y cuyo número de repeticiones es muy variable. Su mayor atractivo reside en su abundancia en el genoma, su herencia mendeliana y su potencial para detectar altos niveles de polimorfismo (Magoulas, 1998). Además se necesita muy poco tejido para hacer los análisis ya que el DNA puede ser amplificado usando la reacción en cadena de la polimerasa (PCR) (Partis y Wells, 1996; Willians, et al., 1998).

Los microsatélites tiene gran utilidad como marcadores individuales para análisis de paternidad y es una herramienta de determinación concluyente en la

determinación del éxito de las manipulaciones genéticas tales como la inducción de la ginogénesis.

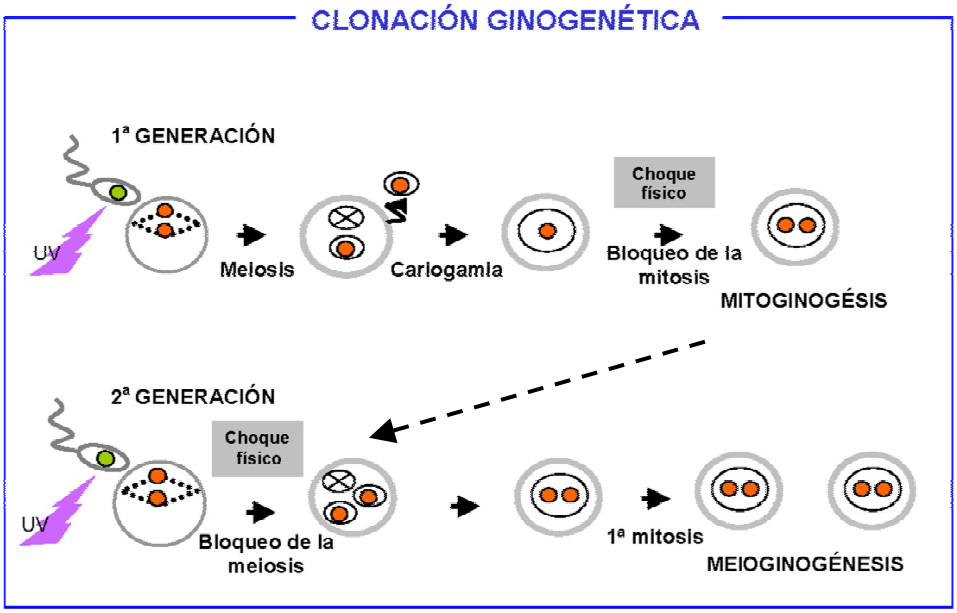

Fig. 5. la clonación ginogenética se alcanza con la manipulación de dos generaciones sucesivas. En la primera se generan individuos mitoginogenéticos con una alta tasa de homocigosis. Los huevos de las hembras de la primera generación se activan con esperma inactivo y se restablece la diploidía bloqueando la extrusión del segundo corpúsculo polar (meioginogeneticos). De esta forma se combina la generación de alta homocigosidad en la primera generación con la mayor viabilidad de los meioginogenéticos respecto a los mitoginogenéticos en la segunda.

Aplicaciones de la ginogénesis y de la androgénesis

Los peces con herencia uniparental se utilizan en investigación aplicada en la acuicultura y en investigación básica (Golovinskaya, 1968; Stanley y Sneed, 1974; Cherfas, 1981). La mayor exigencia en la aplicación de las técnicas de manipulación de gametos de peces marinos es la calidad de los gametos (Wohlfarth, 1990; Bartley, 1998), haciendo así que el éxito de los experimentos sea muy variable dependiendo de los distintos peces utilizados y del estado de los huevos y del esperma. En especies marinas es más complicado que en especies de agua dulce, ya que los huevos son más pequeños y más frágiles (Zohar, 1989), y en muchos casos debido a la condición oligospérmica de los machos.

La primera proposición de la inducción de la ginogénesis en acuicultura, fue como método en el control del sexo (Thorgaard, 1986). En especies en las que las

hembras son el sexo homogamético (hembras XX) y los machos el sexo heterogamético (machos XY), la inducción de ginogénesis normalmente produce descendencia todo hembras (Fig.6A) (Stanley, 1976). Sin embargo hay que mencionar que en algunas especies cuyas hembras son homocigóticas (XX-XY) se han encontrado un variable porcentaje de machos ginogenéticos (Felip et al., 2001a; Devlin y Nagahama, 2002).

La inducción de androgénesis en especies cuyo sexo homogamético son las hembras produce individuos (XX) y supermachos (YY) en la misma proporción. Posteriormente, el cruce de supermachos (YY) con hembras normales (XX), produce poblaciones monosexo masculinas (XY) en la totalidad de la población (Yamamoto, 1975; Parsons y Thorgaard, 1985; Tave, 2003). En especies en las que los machos son el sexo homogamético, hembras (WZ) y machos (ZZ), la androgénesis produce 100% de machos (ZZ) en la primera generación (Fig. 6B). El interés por cultivar poblaciones exclusivamente masculinas, se da en aquellas especies en que o bien el macho crece mejor que la hembra (Mair et al., 1997), o tienen un valor comercial más alto debido a la presencia de algún carácter secundario específico.

Ambas técnicas, son de gran utilidad para la determinación del sexo genético en muchas especies, mediante el análisis de la proporción de sexos en la descendencia (Thorgaard, 1983; Yamazaki, 1983; Ihssen et al., 1990), y son la base fundamental en el desarrollo de métodos de la producción de poblaciones monosexo.

La ginogénesis permite la rápida producción de líneas endogámicas en peces y es particularmente importante cuando se utilizan como madres las ginogenéticas diploides totalmente homocigóticas (mitoginogenéticas) (Strieisinger et al., 1981). Un segundo ciclo de ginogénesis, a partir de huevos de individuos mitoginogenéticos, produciría clones (Fig. 5). La producción de homocigotos facilita la determinación de los parámetros genéticos de cada individuo o población para el desarrollo de mapas genéticos (Nagy y Csany, 1982; Streisinger et al., 1986).

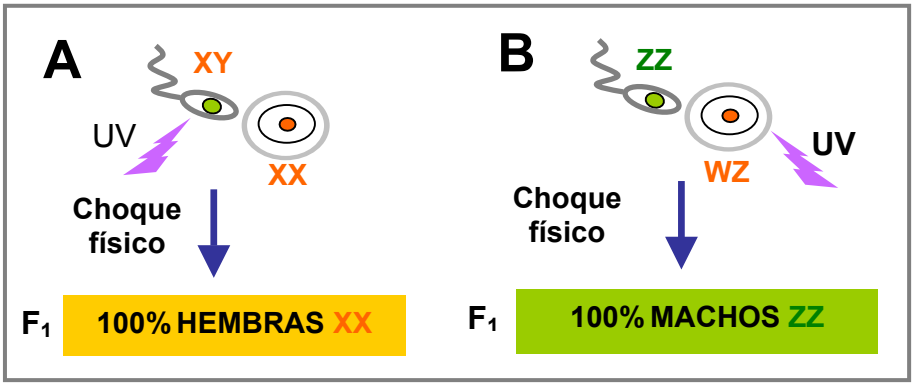

Fig. 6. Inducción de ginogénesis (A) y androgénesis (B). La inactivación del gameto del sexo heterogamético da como resultado la obtención de poblaciones monosexo.

Los ginogenéticos diploides parcialmente homocigóticos (meioginogenéticos), son menos valiosos en la rápida generación de líneas endogámicas, ya que los estudios de heterocigosidad han demostrado que algunos loci permanecen heterocigóticos en una alta proporción en la descendencia (Cherfas y Truweller, 1978; Nagy et al., 1979; Thorgaard, 1983). Esto aparentemente refleja una posición distal de estos loci con relación a su centrómero. Esta información puede ser utilizada en estudios de comparación de la evolución entre genes y entre especies (Thorgaard y Knudsen, 1983). Otra aplicación de la ginogénesis es como transferente de genes en casos en que la inactivación del esperma sea incompleta, y tenga lugar alguna herencia paterna residual (Thorgaard, 1985).

La androgénesis facilita el control de los efectos del genotipo mitocondrial sobre el desarrollo, ya que el DNA mitocondrial en animales es de herencia materna. Una aplicación de evidente interés de la androgénesis es la recuperación de individuos a partir de esperma congelado, sin tener que contar con gametos femeninos de la misma especie o línea genética. Esto es de gran interés para recuperar genotipos de especies protegidas o en extinción.

Tabla 2. Técnicas de manipulación cromosómica en el control del sexo

Gametos	**Esperma + huevos**						
Fertilización	Esperma + Huevos			Esperma irradiado + Huevos			Esperma + huevos irradiados
2ª división meiótica del huevo		Con choque			Con choque		
1ª división mitótica del cigoto			Con choque			Con choque	Con choque
Ploidía	Diploides (2n)	Triploides (3n)	Tetraploides (4n)	Haploides (n)	Meioginogenéticos (2n)	Mitoginogenéticos (2n)	Androgenéticos (2n)
Peces obtenidos	Ambos sexos (fértiles)	Ambos sexos (estériles)	Ambos sexos (fértiles)	Inviables	Heterocigotos (fértiles)	Homocigotos (fértiles)	Ambos sexos (fértiles)

2.2.2. Manipulación fisiológica

El sexo fisiológico en peces puede ser alterado manipulando el proceso de diferenciación sexual, mientras el sexo cromosómico permanece inalterado. Las técnicas de manipulación fisiológica del sexo consisten en la administración de esteroides sexuales durante el proceso de diferenciación sexual. En este proceso, lo primero que se produce es la diferenciación gonadal, y a continuación se desarrollan los caracteres externos y etológicos (Yamazaki, 1983).

En la naturaleza, los esteroides sexuales son inductores de varios fenómenos reproductivos como la diferenciación de las gónadas, la gametogénesis, la ovulación, la espermiación y la puesta, así como de la aparición de caracteres secundarios sexuales, de cambios fisiológicos y morfológicos en la época de puesta, y de la producción de feromonas (Yamazaki, 1983). En la manipulación del sexo fisiológico los esteroides sexuales se utilizan para esterilizar, masculinizar o feminizar, a través de la inducción artificial del cambio permanente del fenotipo sin cambiar el genotipo. Así, el control del sexo gonadal rinde la posibilidad de hacer cultivos de lotes de peces estériles y monosexo, eliminando el sexo no deseado.

El control endocrino del sexo fue iniciado en peces modelo como la medaka, *Oryzias latipes* en los años 50 y 60 (Yamamoto, 1969). Estos estudios derivaron en la aplicación de la tecnología en peces de interés comercial en acuicultura (Hunter y Donaldson, 1983).

A) Control del sexo por tratamiento hormonal

Los esteroides sexuales se administran idealmente antes del periodo de diferenciación sexual, el cual comienza en un tiempo variable después de la eclosión según las especies, antes o después de iniciada la alimentación. La determinación de las variables del tratamiento hormonal en el control del sexo incluyen: tipo de hormona (andrógeno o estrógeno), su naturaleza (natural o sintética), el momento de su aplicación, la dosis, la duración y el número de tratamientos.

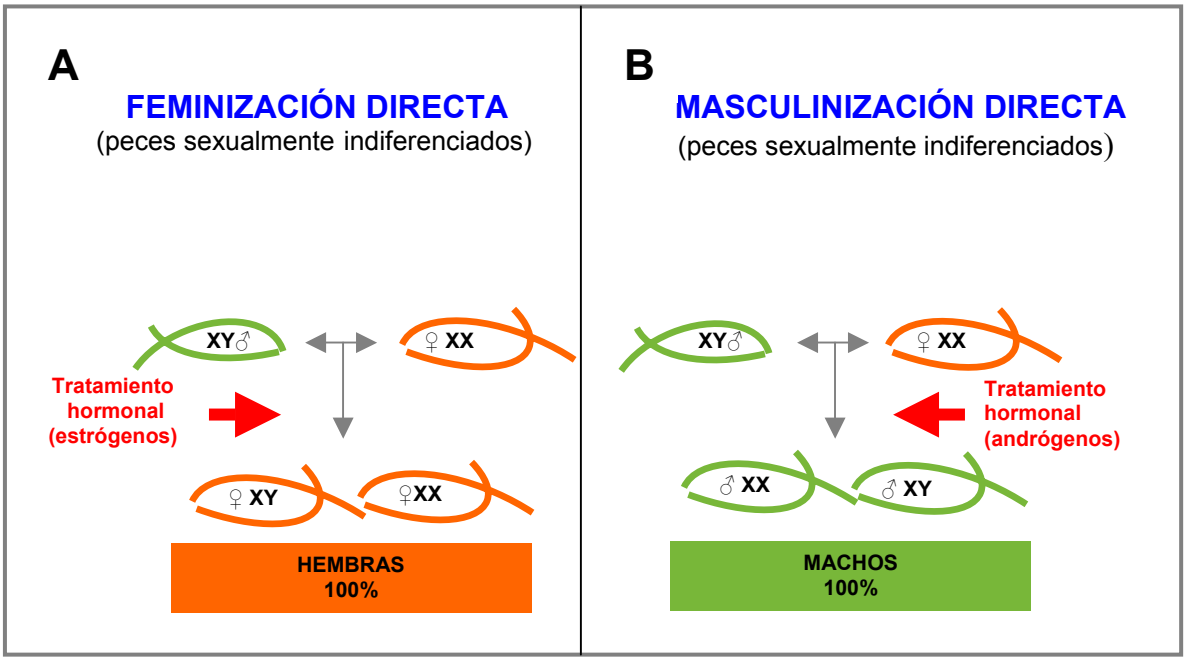

Fig. 7. Métodos directos. Método de feminización directo utilizando tratamiento con estrógenos (A), y de masculinización directo utilizando tratamiento con andrógenos (B).

Las dosis y la duración del tratamiento son específicas de cada especie y pueden ser administrados por medio del alimento o por inmersión cuando la diferenciación sexual comienza antes del inicio de la alimentación (Piferrer, 2001). En vivíparos como el "guppy", *Poecilia reticulata*, la diferenciación sexual es anterior al nacimiento de las crías y entonces la diferenciación debe empezar en el estadio embriónico, tratando a la madre (Takahashi, 1975 a,b)

Los métodos para alterar el sexo por medio de tratamiento hormonal son:

Método directo, que consiste en la masculinización, la feminización o la esterilización por administración durante los primeros estadios de desarrollo del pez de

andrógenos o de estrógenos (McAndrew et al., 1993) (Fig.7). El efecto se consigue ya en la generación tratada y la hormona administrada se elimina en un periodo de semanas (Piferrer y Donaldson, 1984 a; Tave, 2003).

Para inducir feminización se utilizan los estrógenos, siendo el más utilizado el estradiol-17β, y para inducir masculinización se administran andrógenos, siendo el más comúnmente utilizado la 17α-metiltestosterona. Para esterilizar el tratamiento es prolongado y a dosis más elevadas de andrógenos lo que impide que se desarrollen las gónadas, causando esterilidad en los peces. (Piferrer et al., 1994 b). La eficacia y sencillez de su aplicación hace que sea un método muy fácil de utilizar, pero este método puede llevar problemas a la hora de la comercialización por el rechazo del consumidor a peces tratados con hormonas.

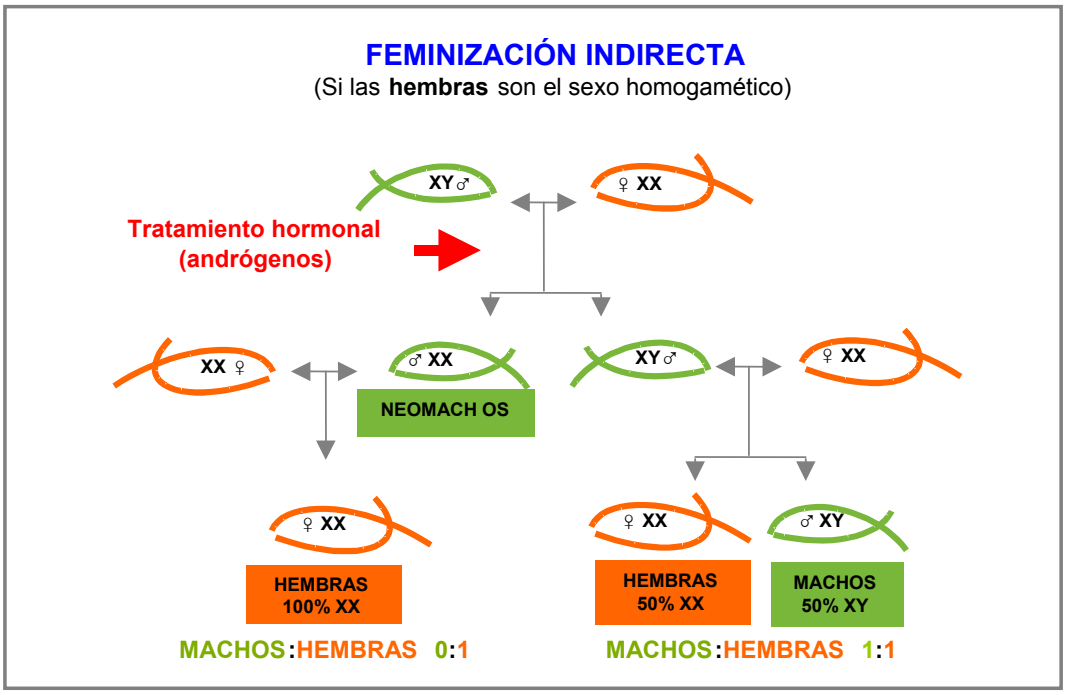

Fig. 8. Métodos indirectos: Feminización indirecta utilizando andrógenos para generar neomachos. Sólo funciona con especies cuyo sexo homogamético sean hembras.

El *método indirecto* consiste en aplicar el tratamiento hormonal en la generación previa para producir gametos monosexos (normalmente esperma). La elección del gameto se hará en función del sexo que se desea producir. Este método tiene la ventaja

de que los peces que se van a comercializar no han sido nunca tratados con hormona (Fig. 8 y 9).

En especies en las que no existen diferencias morfológicas entre sexos como en el rodaballo, la utilización de este método pasa por la necesidad de utilizar el test de progenie, por lo cual son necesarias una o dos generaciones para su implementación.

2.2.3. Manipulación cromosómica y fisiológica combinadas

En la producción de poblaciones todo hembras en algunas especies la utilización de caracteres externos ligados al sexo genético sirven para identificar los neomachos (machos inducidos hormonalmente pero que son genéticamente hembras). En otras especies interesantes como los salmónidos, la lubina o el rodaballo, la determinación del sexo genético en individuos inmaduros presenta muchas dificultades y la única manera de conocerlo es mediante un test de progenie. Este test consiste en determinar la proporción de sexos en la descendencia (F1) de cada cruzamiento con hembras normales. Este método consume mucho tiempo, ya que hay que esperar hasta que estos posibles neomachos alcancen la madurez sexual y sean capaces de reproducirse.

La alternativa a los test de progenie es el uso de sondas genéticas que pueden identificar el sexo, pero estas sondas están sólo disponibles para unas pocas especies (Devlin y Nagahama, 2002).

La feminización a través de la ginogénesis (Refstie et al., 1982), puede solucionar este problema. En especies en las cuales las hembras son el sexo homogamético, la inducción de ginogénesis debería producir en la primera generación individuos genéticamente todo hembras, a las que se puede posteriormente masculinizar para producir neomachos (XX) administrando directamente metiltestosterona a los alevines antes de la diferenciación sexual. Estos neomachos (XX) al cruzarlos con hembras normales darán una descendencia 100% hembras (XX) (Fig. 10).

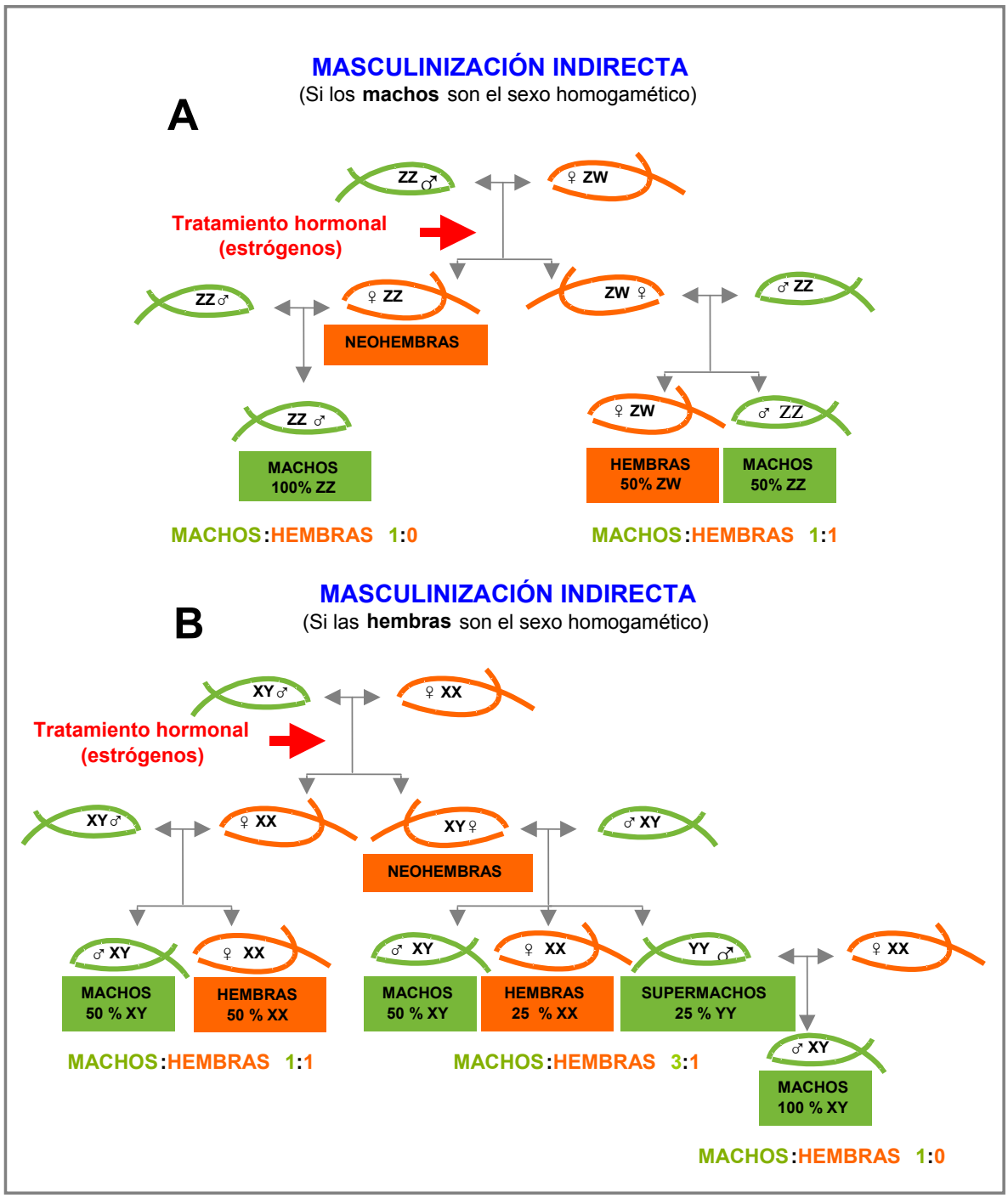

Fig. 9. A y B: Métodos indirectos: Masculinización indirecta en especies cuyo sexo homogamético son los machos (A), con producción de neohembras, y masculinización indirecta en especies cuyo sexo homogamético son las hembras, con la producción de supermachos (B).

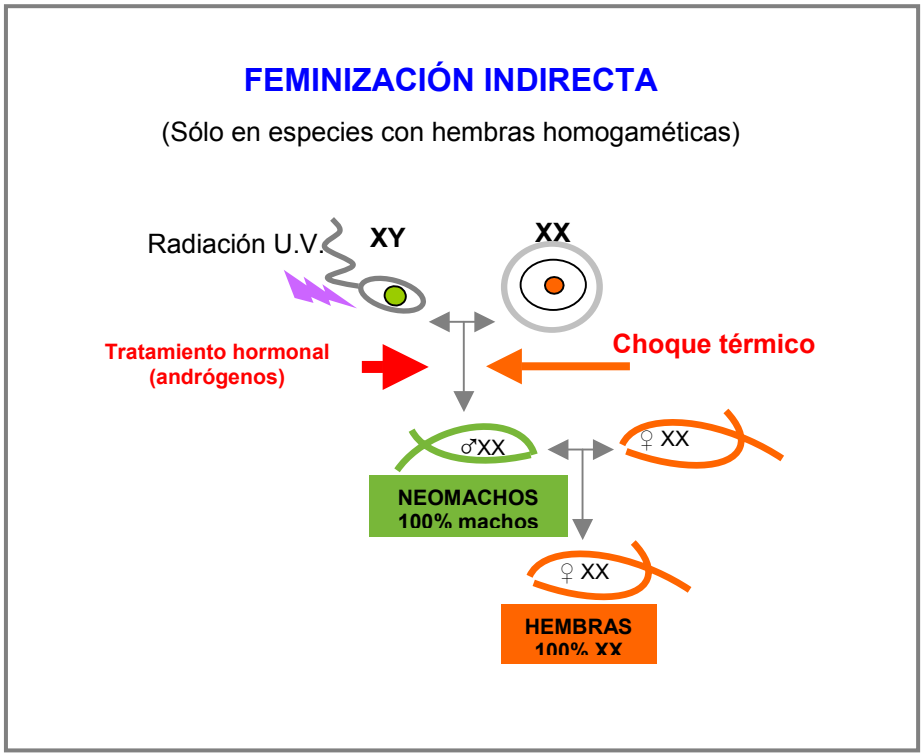

Fig. 10. Feminización indirecta utilizando la inducción a la ginogénesis seguida de un tratamiento hormonal con andrógenos a los alevines para producir neomachos. Estos neomachos ginogenéticos al cruzarse con hembras normales producirán descendencia 100% hembras.

Sin embargo, se ha descrito que hay especies en las que los ginogenéticos no son 100% hembras, obteniéndose un variable porcentaje de machos ginogenéticos (Felip et al., 2001a). En estos casos habría que validar la técnica para determinar el porcentaje de hembras que se pueden obtener.

3. APLICACIÓN DE LAS TÉCNICAS DE MANIPULACIÓN CROMOSÓMICA EN EL CULTIVO DEL RODABALLO

El rodaballo *Scophthalmus maximus* (Linnaeus, 1758), es una especie marina de alto valor comercial. En la naturaleza es poco abundante y su área de distribución se extiende desde Noruega a lo largo de las costas de Inglaterra, Francia, España y Portugal hasta el norte de África, y en el mar Mediterráneo. La temperatura de las costas Atlánticas de Galicia, entre 16 y 18ºC, convierte esta área en una de las más idóneas para su cultivo. España es el primer productor mundial del rodaballo con una producción anual de 4.364 T en el año 2004 (JACUMAR, 2005).

Es una de las especies que mejor cumple los criterios que evalúan las especies para ser cultivadas, como son un alto valor comercial, fácil reproducción, rápido crecimiento, y alimento disponible y barato (Jones, 1972). El conocimiento acerca del cultivo intensivo de esta especie, en lo que se refiere a su reproducción y cultivo, tanto en la fase larvaria como en las fases de engorde, esta bien documentado (Iglesias et al., 1987; Devauchelle et al.,1988; Forés et al., 1990; Sánchez, 1990; Sánchez et al., 1990; Stottrup, 1994; Arnaiz, 1995; Olmedo, 1995; Imsland et al., 1997; Peleteiro, 2001).

El crecimiento durante la etapa juvenil es regular y relativamente rápido y soporta altas densidades de cultivo, alcanzando el segundo año de vida un peso medio entre 1,5 y 2,0 Kg (Sánchez et al., 1990). Es una especie gonocorista que carece de dimorfismo sexual, y las hembras suelen ser más grandes que los machos, lo que se hace evidente sobre todo al alcanzar la primera maduración sexual (Imsland et al 1997). A partir de esta etapa las diferencias de crecimiento se incrementan con el tiempo, entre ambos sexos y provoca que en los lotes se origine una gran dispersión de tamaños con la consiguiente necesidad de desdoblamientos y menor rendimiento de la productividad del cultivo.

El proceso de la reproducción que incluye la maduración sexual y las puestas, abarca varios meses del año y los efectos de la primera maduración sexual se empiezan a mostrar en rodaballos a partir de 20 meses en los machos y en aproximadamente en el 50% de las hembras (Imsland et al., 1997). Como en otras especies de peces teleósteos la primera maduración en rodaballo tiene más que ver con el tamaño que con la edad lo que hace que la cada vez mejor alimentación provoque un mayor crecimiento y en consecuenci, estos procesos de pubertad empiecen a manifestarse a edades más tempranas (Donaldson et al., 1979; Imsland et al., 1997).

La estrategia reproductiva de las hembras de esta especie, consiste en la producción de gran cantidad de huevos (1×10^6 huevos/Kg; Jones, 1974), lo que implica un considerable desarrollo de las gónadas (Devauchelle et al., 1988). Durante esta etapa el pez canaliza toda su energía hacia el crecimiento gonadal, y ralentiza el crecimiento somático (Peleteiro, 2001).

Los efectos no deseados de la maduración sexual no se limitan solamente a ralentización del crecimiento, sino que incluyen falta de apetencia por el alimento, y mayor sensibilidad a temperaturas por encima de 20ºC a bajos niveles de oxígeno, a

enfermedades y a manipulación en general, produciendo mayor mortalidad y pérdida de calidad de las partes comestibles. Todos estos motivos condicionan que en los centros de producción de rodaballo en los cultivos dedicados a la producción, la cosecha de esta especie se realice en los meses previos a la primera maduración sexual antes de que estos síntomas se empiecen a manifestar.

La aplicación de las técnicas de mejora genética permiten ya en algunas especies de interés en acuicultura como el salmón y la trucha, la producción de peces estériles que no maduran y de poblaciones monosexo, favoreciendo la expresión de aquel sexo que lleve asociadas las características que sean más ventajosas para la especie en cuestión, como mayor crecimiento y maduración más tardía (Felip et al., 2001a).

La utilización de tales técnicas en el cultivo del rodaballo, posibilitaría la producción de rodaballos estériles que se puedan cultivar hasta tamaño mayor, sin tener la limitación de la aparición de la primera maduración, eliminándose además los problemas asociados a la reproducción. Por otro lado, la posibilidad de producción de lotes de individuos todo o casi todo hembras que crecen mas que los machos, y que además se pudieran esterilizar, reduciría en gran medida la dispersión de tamaños que normalmente se suceden en las unidades de producción.

La aplicación de estas técnicas genéticas en rodaballo, para producir poblaciones estériles o todo hembras, ha sido el objetivo de esta investigación.

4. OBJETIVOS GENERALES

Basado en lo acabado de exponer, los objetivos globales de esta tesis son la puesta a punto de metodologías para inducir la triploidía y la ginogénesis en el rodaballo, con miras a obtener lotes estériles o monosexo para su aplicación en acuicultura. Los objetivos específicos son, por tanto:

Estudio de la triploidía en el rodaballo:

1) Determinación de las condiciones óptimas para inducir la triploidía en el rodaballo, 2) Verificación de la condición de triploide, 3) Determinación del efecto de la triploidía sobre la composición de la fracción roja de la sangre, 4) Efecto de la triploidía sobre el crecimiento, el desarrollo gonadal, y la proporción de sexos

Estudio de la ginogénesis en el rodaballo:

1) Determinación de las condiciones óptimas para inducir la ginogénesis en el rodaballo, 2) Verificación en los ginogenéticos obtenidos de la herencia exclusivamente materna, 3) Efecto de la ginogénesis sobre el crecimiento, el desarrollo gonadal y la proporción de sexos.

CAPITULO II
LA TRIPLOIDIA EN EL RODABALLO

LA TRIPLOIDIA EN EL RODABALLO

El estudio de la triploidía en el rodaballo fue abordado debido al gran interés que suscita en la acuicultura comercial la esterilidad que la triploidía lleva normalmente asociada.

Este estudio se realizó en dos fases. En la primera, se desarrolló la metodología para la inducción de triploidía en el rodaballo y para el diagnóstico del nivel de ploidía (Artículos 1 y 2). En la segunda, se determinó el efecto de la triploidía sobre la hematología de rodaballos, y sobre la supervivencia, el crecimiento, el desarrollo gonadal y la proporción de sexos en rodaballos desde los 6 meses hasta los 4 años de edad (Artículos 3 y 4).

Resumen: La triploidía en el rodaballo: Inducción, verificación y efectos sobre el crecimiento y la reproducción.

Artículo 1. Piferrer, F., **Cal, R.M**., Álvarez-Blázquez, B., Sánchez, L. and Martínez, P. 2000. Induction of triploidy in the turbot (*Scophthalmus maximus*). I. Ploidy determination and the effects of cold shocks. **Aquaculture**, 188: 79-90.

Articulo 2. Piferrer, F., **Cal, R.M**., Gómez, C., Bouza, C. and Martínez, P. 2003. Induction of triploidy in the turbot (*Scophthalmus maximus*). II**.** Effects of cold shock timing and induction of triploidy in a large volume of eggs. **Aquaculture**, 220: 821-831.

Artículo 3. **Cal, R.M**., Vidal, S., Gómez, C., Álvarez-Blázquez, B., Martínez, P. and Piferrer, F. 2005. Growth and gonadal development in diploid and triploid turbot (*Scophthalmus maximus*). **Aquaculture** (en prensa).

Artículo 4: Cal, R.M., Camacho, T., Vidal, S., Piferrer, F. and Guitián, F. J. 2005. Effect of triploidy on turbot haematology. **Comparative Biochemistry and Physiology, Part A,** 141, 35-41.

RESUMEN

LA TRIPLOIDÍA EN EL RODABALLO: INDUCCIÓN, VERIFICACIÓN Y EFECTOS SOBRE EL CRECIMIENTO Y LA REPRODUCCIÓN.

El cultivo del rodaballo está firmemente consolidado en Europa con una producción de 6080 t en 2004 (FEAP, 2005). Durante el cultivo intensivo, las hembras crecen notablemente más que los machos siendo esta diferencia asociada al sexo una de las mayores descritas en cultivos marinos (Piferrer et al., 1995). Las diferencias de crecimiento implican un mayor número de desdoblamientos para optimizar los regímenes de alimentación. Por otra parte, la maduración sexual normalmente se produce en los machos y con frecuencia en las hembras antes de alcanzar el tamaño comercial, lo que reduce el crecimiento somático e incrementa la mortalidad. Todo ello tiene como consecuencia una pérdida económica durante la fase de crecimiento, y por este motivo existe un gran interés por producir lotes de todo hembras o estériles.

En muchas especies se han obtenido lotes de peces estériles mediante manipulación cromosómica induciendo la triploidía (Thorgaard, 1983; Benfey, 1989). Recientemente, la manipulación cromosómica se ha aplicado a peces marinos de importancia en la acuicultura europea, como la dorada, *Sparus aurata* (Gorshkova et al., 1995; Garrido-Ramos et al., 1996) y la lubina, *Dicentrarchus labrax* (Carrillo et al. 1993; Colombo et al., 1995; Gorshkova et al., 1995; Felip et al., 1997). La triploidía y la ginogénesis son fenómenos que en algunas especies de peces pueden aparecer de forma natural (Thorgaard, 1983). En Europa, según la legislación vigente de la Unión Europea, los peces en los que se ha inducido la triploidía (es decir, que tienen un conjunto extra de cromosomas en el núcleo de sus células) no se consideran organismos genéticamente modificados.

A pesar de su importancia en Europa, para el rodaballo no hay ninguna información publicada acerca de la manipulación cromosómica. Para inducir la triploidía en el rodaballo manteniendo la mayor supervivencia posible, se utilizó un planteamiento similar al utilizado en la lubina (Felip et al., 1997). Este protocolo consistió en probar diferentes choques térmicos examinando los resultados en términos de tasas de supervivencia y de triploidía obtenidas.

La triploidía fue inducida en el rodaballo mediante la aplicación de choques fríos poco después de la fertilización (DF) y se investigaron los efectos combinados de: el momento de inicio del choque frío tras la fertilización, la duración del choque y la temperatura del mismo.

Los objetivos de este estudio fueron, en primer lugar, optimizar las tres variables fundamentales del choque térmico, validar el uso del análisis de NOR para comprobar los niveles de ploidía en esta especie y determinar y cuantificar los efectos de la triploidía sobre el crecimiento y la reproducción de los rodaballos triploides.

Los gametos se obtuvieron durante los meses de abril-junio del lote de reproductores de rodaballos criado en las instalaciones del Centro Oceanográfico de Vigo (NO de España). Sesenta días antes de su uso, se modificó el fotoperiodo y se ajustó a uno constante de 16 horas de luz: 8 horas de oscuridad y a una temperatura del agua constante de 13-14ºC para estimular la maduración y la puesta.

La triploidía se indujo mediante aplicación de choques fríos poco después de la fertilización. Los choques fríos comenzaron 5 min después de la fertilización y se investigaron diferentes combinaciones de tres temperaturas (0, 2 o 4ºC) y cuatro duraciones de choque (5, 10, 20 y 40 min), produciendo 12 grupos tratados distintamente.

Este experimento fue repetido varias veces. El conjunto de huevos se dividió en 15 grupos: tres de control y 12 tratados (choque frío). Cada grupo se hizo por cuadriplicado. Se utilizaron tres tipos de grupos de controles: un grupo de control no tratado formado por huevos inmediatamente transferidos a los incubadores (véanse detalles a continuación) sin manipulación después de la fertilización ni aclarado alguno. Este grupo sirvió como control de calidad de los gametos y del procedimiento de fertilización artificial. Los otros dos grupos fueron controles *sham,* formados por huevos sometidos a la misma manipulación a la que se sometieron los grupos a los que se les aplicó choque frío (vertidos en distintos viales y manipulados), con la diferencia de que se mantuvieron constantemente a temperaturas de 13–14ºC. Estos controles sirvieron para evaluar el efecto de la manipulación mecánica inmediatamente después de la fertilización. Un grupo de control *sham* se transfirió a los incubadores 10 min después de la fertilización sin más manipulación y el otro a los 40 min después de la fertilización, coincidiendo con dos de los cuatro tiempos de duración de los choques fríos (véase abajo).

Cuando los choques fríos se aplicaron 5 min después de la fertilización durante 5, 10, 20 o 40 min, a temperaturas de 0, 2, o 4ºC , los resultados indicaron que el número de triploides aumentaba con temperaturas de choque más bajas y con una mayor duración del choque en el rango de 5 a 20 min. En concreto, los choques fríos de 0ºC aplicados durante 20 min, dieron sistemáticamente como resultado un ~90% de rodaballos triploides ($P < 0.001$), y una supervivencia de ~80% con respecto a los controles no tratados. Los choques con una duración superior a 20 min (40 min) no aumentaron el número de rodaballos triploides, en contraste con lo encontrado en el caso de otras especies de peces planos. Esto está probablemente relacionado con la mayor temperatura de prechoque a la que se incuban los huevos de rodaballo. Además, la supervivencia disminuyó con la duración del choque desde el 82-97% en choques de 5 min hasta el 60-70% en choques de 40 min.

Cuando los choques se iniciaron entre 6 y 7 min después de la fertilización, con una temperatura previa al choque de 13–14ºC, se obtuvo un pico claro en la tasa de inducción de la triploidía. Con estos tiempos, los choques de 20 min de duración a 0ºC produjeron > 90% de triploidía, con una supervivencia de aproximadamente el 80% respecto de los controles no tratados. Esta supervivencia no fue significativamente distinta (P > 0,05) de los controles *sham*, indicando que la menor supervivencia se debe a los efectos de la manipulación y al estrés durante la inducción de la triploidía más que a la condición de triploidía en sí.

Se realizaron experimentos con pequeños y grandes volúmenes de huevos con el fin de determinar cómo podrían afectar a la supervivencia y la inducción de la triploidía los cambios en los volúmenes relativos de los huevos y el agua fría. Para garantizar altas tasas de triploidía y de supervivencia, fue necesario mantener cuidadosamente la temperatura del agua justo por debajo de 0ºC. Finalmente se concluyó que la mejor combinación para inducir la triploidía en el rodaballo es la siguiente: comienzo del choque 6.5 min después de la fertilización, duración del choque 25 min y temperatura del mismo entre 0 y –1ºC. Con esta combinación, podía inducir sistemáticamente un 100% de triploidía con una supervivencia media del 60% respecto de los controles no tratados. Esto se aplicó con éxito a un gran volumen de huevos (~ 300 ml; 1 ml ~ 800 huevos) con el fin de producir rodaballos triploides en masa. Los triploides registraron una menor tasa de supervivencia que los diploides en la eclosión, pero similar después de la misma, con la capacidad de completar las distintas fases del desarrollo larvario, lo que indica la viabilidad de producir rodaballos triploides en condiciones de cultivo.

En nivel de la ploidía se evaluó mediante el recuentro del número de regiones de organización nuclear (NOR) en las larvas y también mediante la medición del tamaño de los eritrocitos en los juveniles. Para controlar los efectos del choque frío en especies nuevas, el análisis de las regiones de organización

nuclear (NOR) es una técnica rápida, fiable y barata que determina el nivel de ploidía incluso en células embrionarias (Phillips et al., 1989).

Dado que esta especie muestra polimorfismo en el número de regiones organizadoras del nucleolo (NOR), se validaron en primer lugar las determinaciones del nivel de ploidía a través de análisis de NOR. Los resultados demostraron que el análisis de NOR podría discriminar bien entre individuos diploides y triploides cuyo nivel de ploidía se verificó cariotipicamente (n=44 cromosomas en 2n; n = 66 cromosomas en 3n). En los diploides, el número medio de NOR por célula varió entre 1,10 y 1,85, mientras que en los triploides varió entre 1,50 y 2,35. Sin embargo, el histograma de distribución de los datos del número medio de NOR por célula mostró que el número de peces en la región de solapamiento (número de NOR entre 1,50 y 1,85) fue muy bajo. Los peces que habían sido sometidos a choque frío con valores de NOR > 1,735 se consideraron triploides. Se observó que el error en el cálculo de la ploidía en el rodaballo mediante análisis de NOR se situó sistemáticamente alrededor del 3% y fue siempre < 5%. De este modo, el análisis de NOR podría aplicarse de forma segura en el control del efecto de los choques fríos sobre la inducción de la triploidía.

Morfológicamente, los peces triploides no se pueden distinguir de sus correspondientes controles diploides. Sin embargo, a causa de tener un conjunto extra de cromosomas, la triploidía causa un incremento en el tamaño celular con una concomitante reducción en el número de células en una variedad de tipos, incluyendo los eritrocitos (Benfey, 1999). Sin embargo, el mecanismo compensatorio que posibilita esta reducción todavía hoy no es bien conocida.

Los índices morfométricos de los eritrocitos fueron determinados en extensiones de sangre por microscopia óptica, observándose que la triploidía incrementa significativamente ($P<0.001$) todos los índices morfométricos medidos en los eritrocitos, incluyendo tamaño, superficie y volumen, excepto el tamaño del eje menor nuclear ($P>0.05$). El incremento en tamaño celular fue más grande para

el eje mayor (31.0%) que para el eje menor (8.3%), resultando así los eritrocitos triploides más elipsoidales. El incremento en el volumen de los eritrocitos (45.9%) fue próximo al incremento teóricamente esperado (50%) como resultado de un conjunto extra de cromosomas. También se observó el aumento de anormalidades como la presencia de núcleos segmentados y de micronúcleos.

Los índices hematológicos fueron medidos automáticamente con un Coulter Counter hematológico. Se observó que los rodaballos triploides tienen menor número de eritrocitos (NE: 1.84 cel.pl^{-1} en 2n vs. 1.27 cel. pl^{-1} en 3n; $P< 0.001$) pero más grandes (Volumen corpuscular medio [VCM]: 145.51 fl en 2n vs. 181.78 fl en 3n; $P< 0.001$). Sin embargo, la disminución en NE no fue compensada por el incremento en el VCM y por este motivo la triploidía disminuyó el hematocrito (Htc: 26.80% en 2n vs. 23.11% en 3n; $P< 0.001$) y la concentración de la hemoglobina total en sangre (Hb: 73.74 $g.l^{-1}$ en 2n vs. 67.54 $g.l^{-1}$ en 3n; $P< 0.05$). Por contra, la cantidad de hemoglobina media corpuscular (HMC: 40.27 pg en 2n vs. 53.28 pg en 3n; $P< 0.001$) fue más alta en los rodaballos 3n que en los 2n como resultado del mayor tamaño de sus eritrocitos, aunque la concentración media corpuscular (CMC: 0.28 $pg.fl^{-1}$ en 2n vs. 0.29 $pg.fl^{-1}$ en 3n; $P> 0.05$) no fue significativamente diferente.

El NE, Hct y VCM fueron también determinados por métodos manuales. En general, las discrepancias entre los dos métodos fueron pequeñas (aprox. 7%) tendiendo el Coulter Counter a sobreestimar NE y Htc (y, en consecuencia, a subestimar el VCM). Sin embargo, las diferencias entre ploidías fueron muy similares en las determinaciones realizadas con ambos métodos, confirmando así que los cambios en las características hematológicas estaban asociados a la triploidía.

El aumento del volumen asociado a la disminución del número de eritrocitos reduce la proporción superficie/volumen, lo que junto con la disminución del número de eritrocitos, reduce el área total disponible para la

asimilación del oxigeno. Esto puede afectar también a otros procesos importantes como el intercambio iónico y de nutrientes. El cambio de forma observado tanto en la célula como en su núcleo, puede afectar también la asimilación del oxígeno debido al aumento de las distancias de difusión dentro del eritrocito (Benfey, 1999).

Al ser los eritrocitos responsables del transporte de oxigeno desde el medio externo a la célula, cualquier alteración de su número y tamaño puede potencialmente comprometer la habilidad de los triploides para utilizar oxigeno. Ello puede no ser siempre aparente pero puede ser de relevancia en ciertas circunstancias, e.g., bajo condiciones de cultivo subóptimas debido, por ejemplo, a una alta temperatura del agua o excesiva densidad de cultivo. En consecuencia, una menor capacidad para utilizar oxígeno puede reducir el bienestar de los peces y por lo tanto su apetito y nivel de inmunidad. Estudios previos han mostrado, que los peces triploides pueden ser más susceptibles al estrés asociado a la práctica del manejo rutinario en granjas así como a las enfermedades (Benfey and Sutterlin, 1984; Aliah *et al*., 1991). Trabajos preliminares con el rodaballo han puesto de manifiesto que los triploides tienen menor habilidad para reaccionar a la hipoxia aguda que sus homólogos diploides (Cal *et al*., 2001).

El conocimiento de estas alteraciones, es importante cuando se trata de evaluar la viabilidad de los rodaballos triploides en sistemas de cultivo intensivo, en los que pueden darse situaciones ambientales desfavorables, como son la baja concentración de oxígeno.

El efecto de la triploidía sobre el crecimiento y el desarrollo gonadal se determinó en cuatro grupos de peces, dos diploides y dos triploides, desde los 6 a los 48 meses de edad, procedentes de una misma puesta y que fueron cultivados separadamente bajo las mismas condiciones.

Durante el experimento todos los peces fueron muestreados cada dos meses en longitud y peso, determinándose las tasa de crecimiento en cada periodo

y el índice de condición. Periódicamente, se sacrificaron muestras de ambas ploidías y se extrajeron las gónadas para determinar el IGS y ser analizadas histológicamente.

Casi todos los peces del experimento, (n=144 diploides) y (n=150 triploides) fueron sexados. En diploides la proporción de sexos fue 1M:0.6H, mientras que en triploides fue 1M:3.3H.

Todos los peces triploides fueron estériles. Las gónadas de los machos triploides fueron similares aunque más pequeñas que las de los diploides, mientras que las de las hembras triploides fueron significativamente menores y rudimentarias. Los análisis histológicos realizados a los 47 meses de edad mostraron una esterilidad completa en ambos sexos.

Durante la etapa juvenil, la supervivencia fue similar en ambas ploidías ($P>0,05$), sin embargo después de la primera maduración sexual, la supervivencia fue del 91,9% en diploides y del 100% en triploides, ya que debido a la esterilidad de estos últimos se evitaron las mortalidades asociadas a los periodos tras las puestas.

Durante la fase de la pubertad, la triploidía no representó ninguna ventaja en términos de ganancia en longitud y peso, pero a partir de la primera maduración sexual (~ 24 meses, 1600 g) y hasta el final del experimento, el peso medio de los triploides fue ligeramente mayor que el de los diploides, con significativas diferencias durante los periodos tras la puesta debido a la disminución en peso en los diploides, especialmente en las hembras, a causa de la regresión gonadal. Por el contrario, debido a la esterilidad de los triploides, especialmente en las hembras, esta reducción casi no fue apreciada, y por eso los triploides crecieron de forma regular. Las diferencias desaparecieron cuando el nuevo ciclo sexual comenzó debido al rápido incremento en peso de las gónadas

de los peces diploides. Desde los 24 a los 48 meses de edad los triploides mostraron un peso medio aproximadamente 11,4% superior al de los diploides.

El mayor número de hembras en los triploides representó una ventaja adicional, ya que como en los diploides (Imsland et al.,1997), también en los triploides las hembras crecieron más en peso que los machos. El alto porcentaje de hembras en los triploides tuvo una ventaja adicional en términos de biomasa durante el tercero y cuarto año de vida. La biomasa de los triploides fue el 10,3% o 14,3 % mayor que la de los diploides según se considere el peso total o el eviscerado, respectivamente.

En resumen, el cultivo de rodaballos triploides presenta una mayor proporción de hembras que crecen más que los machos. Además, las hembras triploides son estériles, por lo cual crecen constantemente, evitando la reducción en el crecimiento que tiene lugar durante los periodos de puesta, y evitándose también las mortalidades que se suceden en los periodos tras la puesta, facilitando la comercialización en cualquier época de año. Además, el alto porcentaje de hembras reduce la dispersión que normalmente se produce en los diploides, debido a que unos peces maduran y otros no, así como a la diferencia natural de peso entre machos y hembras.

El conjunto de todas estas observaciones indica que la inducción de la triploidía puede ser una opción interesante en la acuicultura del rodaballo, especialmente para la producción de peces de tamaño grande de más de 1,5 Kg.

Artículo 1

Induction of triploidy in the turbot (*Scophthalmus maximus*). I. Ploidy determination and the effects of cold shocks

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Induction of triploidy in the turbot (*Scophthalmus maximus*) I. Ploidy determination and the effects of cold shocks ☆

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Abstract

The basis for induction of triploidy in the turbot by applying cold shocks shortly after fertilization (AF) was studied. Since this species exhibits a polymorphism in a number of nucleolar organizing regions (NOR), determination of the ploidy level through NOR analysis was first validated. Results showed that NOR analysis could discriminate well between diploid and triploid individuals whose ploidy level was verified karyotypically ($n = 44$ chromosomes in diploids; $n = 66$ in triploids). In diploids, the mean number of NOR per cell ranged from 1.10 to 1.85, whereas in triploids, it ranged from 1.50 to 2.35. However, histogram distribution of data on mean number of NOR per cell showed that the number of fish in the overlapping region (NOR number between 1.50 and 1.85) was very low. Cold-shocked fish with a NOR value > 1.735 were considered triploids. The error in ploidy assessment using NOR analysis in the turbot was found to be consistently around 3% and always $< 5\%$. In this way, NOR analysis could be safely applied to monitor the effects of cold shocks on triploidy induction. Cold shocks were applied 5 min AF for 5, 10, 20 or 40 min at either 0°C, 2°C, or 4°C. Results showed that the number of triploids

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increased with lower shock temperatures and longer shock duration in the range from 5 to 20 min. In particular, cold shocks of 0°C applied during 20 min consistently resulted in ~90% triploid turbot ($P < 0.001$). Shocks longer than 20 min (40 min) did not increase the number of triploid turbot in contrast to what has been found in other flatfish species. This is probably related to the higher pre-shock temperature at which turbot eggs are incubated. Survival, 1 day after hatching, was ~80% of the untreated controls and not different ($P > 0.05$) from appropriate sham controls, indicating that lower survival is due to the effects of mechanical handling and stress during triploidy induction rather than the triploid condition per se. The highest triploid yield obtained in this study, ~70%, is higher than the triploid yield obtained in other flatfishes.

Keywords: Sex control; Triploidy; Cold shocks; NOR; Turbot; *Scophthalmus maximus*

1. Introduction

Turbot culture is firmly established in Europe with a production of 2966 t in 1995 (FAO, 1997). During intensive farming, females markedly outgrow males. This sex-related difference is among the largest recorded in cultured marine fish (Piferrer et al., 1995). In addition, sexual maturation usually occurs in males and often in females before they reach marketable size. Differential growth implies a higher number of fish sortings to optimize feeding regimes. Sexual maturation reduces somatic growth and increases mortality. Together, this results in an economic loss during the growout phase. There is, therefore, great interest to produce all-female or sterile stocks to avoid these problems.

All-female or sterile fish have been obtained in many species by chromosome set manipulation to induce gynogenesis and triploidy, respectively (Thorgaard, 1983; Benfey, 1989). Triploidy and gynogenesis are both naturally occurring phenomena in fish (Thorgaard, 1983). Fish, in which triploidy has been induced (i.e., they have an extra set of chromosomes in the nucleus of their cells), are not considered genetically-modified organisms in Europe and elsewhere. Recently, chromosome set manipulation has been applied to marine fishes important for European aquaculture including the sea bream, *Sparus aurata* (Gorshkova et al., 1995; Garrido-Ramos et al., 1996) and the sea bass, *Dicentrarchus labrax* (Carrillo et al. 1993; Colombo et al., 1995; Gorshkova et al., 1995; Felip et al., 1997). To the best of our knowledge, no data have been published regarding chromosome set manipulation in turbot despite its importance in European aquaculture (FAO, 1997). This is interesting considering that the pioneering works of Purdom (1972) on chromosome set manipulation were made in flatfish. The induction of triploidy and gynogenesis has been recently reported for some flatfish species: the olive flounder, *Paralychthys olivaceus* (Tabata, 1991; Kim et al., 1993); the common sole, *S. solea* (Howell et al., 1995); and the halibut, *H. hippoglossus* (Brown et al., 1997; Holmefjord and Refstie, 1997). Generally, cold shocks have been used to induce triploidy in these species.

Turbot is known for having lower larval survival than many other cultured marine fish (Devauchelle et al., 1988). Thus, to induce triploidy in the turbot while maintaining the highest possible survival, an approach similar to that employed in the sea bass (Felip

et al., 1997) was used. This approach consisted of testing different cold shocks and examining their bearing on both survival and triploidy. To monitor the effects of cold shocks in a new species, analysis of the nucleolar organizing regions (NORs) is a fast, reliable and inexpensive technique for determining ploidy, even in embryonic cells (Phillips et al., 1989). Thus, for a given species in which diploid fish exhibit 1 or 2 NOR per nucleus, treated fish with up to 3 NOR are considered triploids. The turbot has a maximum of 2 NOR per nucleus (Bouza et al., 1994). However, a recent molecular cytogenetic study has revealed the existence of a polymorphism in the number of NOR, although it affects only a few individuals which exhibit 3 NOR per nucleus (Pardo et al., 1998). This polymorphism could cause difficulties in the application of NOR analysis in ploidy determination in turbot.

Therefore, the objectives of this study were, firstly, to investigate the effects of temperature and duration of cold shocks on survival and triploidy rates in the turbot, and secondly, to validate the use of NOR analysis to check ploidy levels in this species.

2. Materials and methods

2.1. Gamete collection and artificial fertilization

Gametes were obtained during April–June from turbot broodstock reared at the facilities of the Centro Oceanógrafico de Vigo (NW Spain). Sixty days before use, broodstock were switched to a constant photoperiod of 16 h of light:8 h of darkness and to a constant water temperature of 13–14°C to stimulate the onset of maturation. For each trial, eggs from one to two ovulated females and milt from two to four running males were obtained by abdominal massage. Egg quality (egg diameter $\sim$ 1.1 mm; 1 ml of eggs $\sim$ 800 eggs) was assessed according to the criteria of McEvoy (1984). Since turbot are poor milt producers in terms of both quality and quantity compared to other marine teleosts (Suquet et al., 1994), an excess of milt ($\sim$ 200 μl of milt for each 10 ml of eggs) was used for artificial fertilization. First, eggs were coated with milt in the proportion described above. Then, each volume of eggs plus milt was mixed with 2 vol of seawater. This triggered sperm motility, and thus, fertilization. This moment was considered time zero. Thirty seconds after fertilization (AF), eggs were gently rinsed with excess seawater for 30 s and then divided into groups of approximately 500 each ($\sim$ 100 eggs/ml of water) and kept in clean 20 ml glass vials until use. The seawater used to fertilize and rinse the eggs was at the same temperature as the seawater in which the broodstock were maintained, i.e., 13–14°C. No attempts were made to separate viable and non-viable eggs. Viability was assessed in excess fertilized eggs not needed for the trials by placing them in a graduate cylinder, letting them sit for about 5 min and measuring the proportion that floated. As a precaution, unshocked egg batches with less than 50% survival after 24 h were discarded.

2.2. Cold shock application and experimental design

This study consists of one experiment repeated several times (trial). For each trial, the eggs of one to two females were used. Pooled eggs were divided into 15 groups: three

controls and 12 treated (cold-shocked). Each group was carried out in quadruplicate. Three types of control groups were used. An untreated control group consisted of eggs that were immediately transferred to the incubators (see details below) with no further disturbance AF and rinsing. This group served as a control for gamete quality and artificial fertilization procedures. The other two remaining control groups were sham controls consisting of eggs subjected to the same manipulations to which cold shocked eggs were subjected (pouring into different glass vials and handling), except that they were always maintained at 13–14°C. These controls served to evaluate the effects of possible mechanical disturbances shortly AF. One sham control group was transferred to the incubators and left undisturbed 10 min AF and the other 40 min AF, coinciding with two out of the four durations of the cold shocks (see below).

Triploidy was induced by applying cold shocks shortly AF. For cold shocks, each one of the 12 treated groups of approximately 500 eggs ($\sim$ 5 ml) was poured to another glass vial containing approximately 10 ml of seawater pre-chilled at the desired temperature. These vials were attached to the bottom of a plastic tray filled with a mixture of crushed ice and seawater in such a way that it almost covered the glass vials. Actual water temperature inside the vials fluctuated ± 0.5°C as determined with a precision thermometer graduated in steps of 0.1°C.

Cold shocks started 5 min AF and different combinations of three shock temperatures (0°C, 2°C, or 4°C) and four shock durations (5, 10, 20 or 40 min) were investigated, giving rise to 12 different treated groups.

Control and treated groups were incubated in plexiglass cylinders (15 cm diameter, 3 l capacity) fitted with a bottom mesh (300 μm pore) partially submerged inside a large tub provided with recirculated, filtered, UV-sterilized, and aerated seawater thermoregulated at 13–14°C. Since each one of the three control and each one of the 12 treated groups was carried out in quadruplicate, there were therefore a total of 60 incubators with eggs and larvae in each trial.

2.3. Calculation of survival and ploidy determination

Under the incubation conditions described above, hatching typically took place over 1 day, 5 days AF. The non-fertilized eggs, non-hatched eggs, and larvae were counted and added to obtain the total number of eggs initially used in each replicate of each group. Survival was calculated 1 day after hatching as the number of live larvae with respect to the number of initial eggs and was expressed as a percentage.

Ploidy was determined in larvae collected 1 day after hatching. For this procedure, a sample of larvae from each group in each experiment were kept swimming in a solution of 0.005% colchicine for 6 h. Metaphase spreads were obtained following the technique of Kligerman and Bloom (1977). Slides were stained with silver nitrate as previously described (Howell and Black, 1980). Ploidy was determined by counting the number of NOR in the nucleus of 50 cells from each larvae ($n = 32$ larvae per group) and verified by direct counting the number of chromosomes in five to 10 good metaphase plates in $\sim$ 20% of the examined larvae. In this manner, the mean number of NOR per cell was correlated with its true ploidy level as assessed by karyological analysis for about seven larvae per group in each trial.

2.4. Statistical analysis of data

Only trials in which actual survival 1 day after hatching was $> 50\%$ in the untreated controls were used. Thus, the data presented were obtained from three successful, separate trials with eggs from different females. Survival at 1 day after hatching was transformed to percentages and expressed relative to the survival of the untreated control, whose survival was set at 100% (Volckaert et al., 1994; Felip et al., 1997). Percentage data were arcsin transformed before analysis of variance (ANOVA). Data are expressed as mean $\pm$ SEM.

3. Results

Karyological analysis showed that 1 day after hatching, all metaphase spreads of larvae from both control and sham control groups had 44 chromosomes (Fig. 1A). In

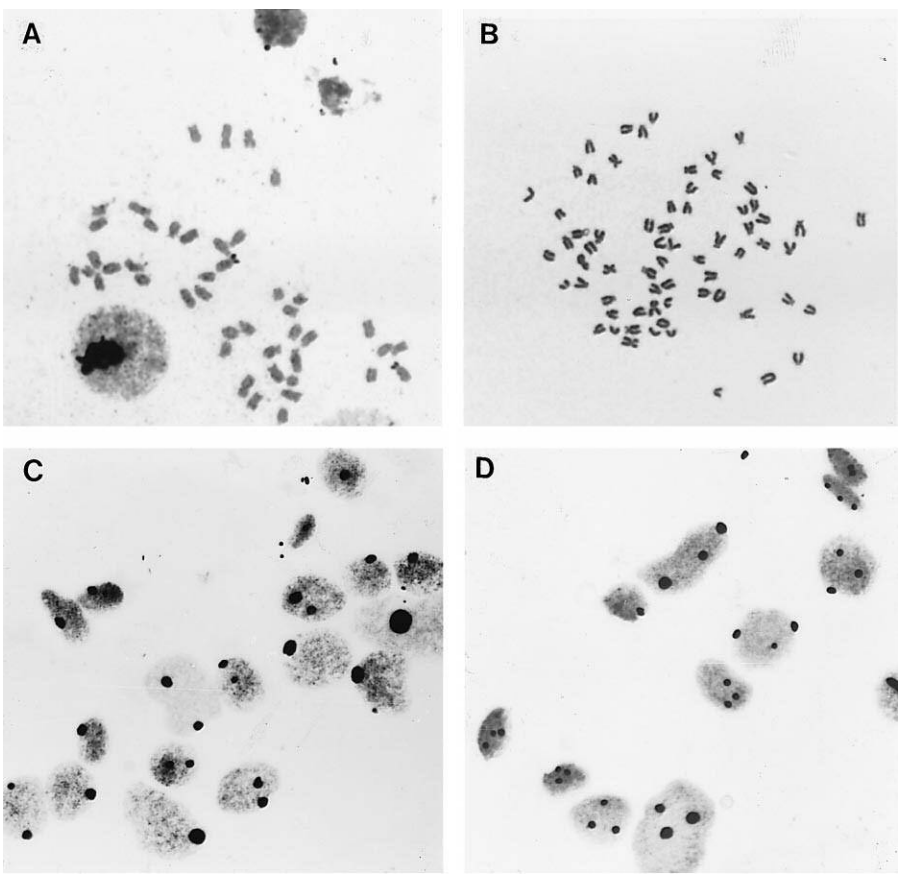

Fig. 1. Ploidy identification in turbot. Typical karyotypes and nuclei of cells obtained from diploid (A and C, respectively; $2n = 44$) and triploid (B and D, respectively; $3n = 66$) turbot larvae at 1 day after hatching and analysed in this study.

contrast, cold shocked groups had a variable proportion of individuals exhibiting 66 chromosomes (Fig. 1B), depending on which treatment conditions they had been subjected to (see below). These fish were considered triploids. No aneuploid or mosaic individuals were observed.

Examination of Ag-stained slides revealed that the average number of active NOR per nucleus of cells from diploids ranged from 1.10 to 1.85 (Fig. 1C). In nuclei from larvae that were triploids as confirmed by direct counting of the number of chromosomes, this value ranged from 1.50 to 2.35 (Fig. 1D). There was therefore an overlapping range from 1.50 to 1.85. However, when a frequency histogram of mean number of NOR per nucleus of cells from larvae whose ploidy level was verified karyotypically was constructed, a clear differentiation of NOR distributions between ploidies was observed (Fig. 2). Percentiles were calculated from these distributions at the 99% confidence interval. Thus, on average, the mean NOR number per cell that comprised 99% of cells in the diploids (right tail) was 1.84 and the mean NOR number per cell that comprised 1% in the triploids (left tail) was 1.63. The mid point between 1.64 and 1.84 was 1.735 and was used as the threshold average value in the number of NOR (dotted line in Fig. 2).Thus, larvae in the treated groups with an average number of NOR > 1.735 were considered triploids. This value was found to range from 1.70 to 1.77 in initial trials and was used subsequently to discriminate between diploids and triploids. Since the number of diploid larvae that were above 1.735 was very low (see Fig. 2), the error of classifying a treated larvae as being triploid when in fact it could be diploid (i.e., the larvae in the control groups with mean NOR > 1.735; see Fig. 2) was

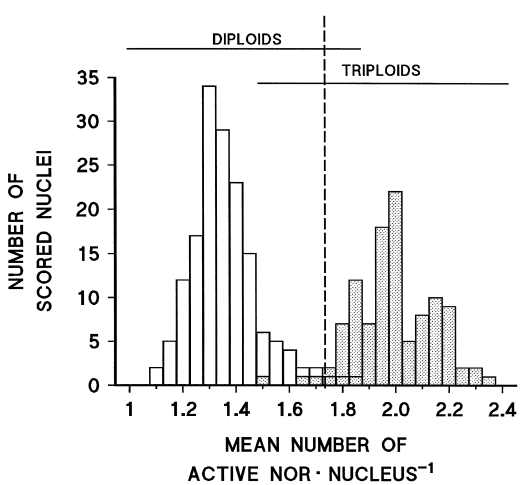

Fig. 2. Frequency histogram of mean number of active NORs per nucleus of cells from individual larvae whose ploidy level was verified karyotypically. Open bars: control diploids; shaded bars: triploids. Note the clear differentiation of both NOR distributions, but also the existence of an overlapping area. The dotted vertical line indicates the threshold average in number of NOR (1.735) used to discriminate between diploid and triploid larvae. About three fish in a 100 would be misclassified. Data from two separate trials. See text for further details.

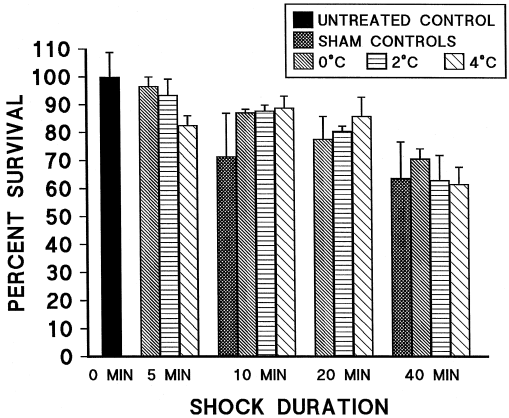

Fig. 3. Relationship between shock temperature and shock duration with survival at 1 day after hatching. Shocks of 0–4°C were applied during 5–40 min, starting 5 min AF. Survival expressed relative to the untreated control, set at 100%. See Section 2 for a detailed explanation of the different types of controls used. Data as mean + SEM of three separate trials, with each treatment in each trial carried out in quadruplicate.

consistently calculated to be $< 5\%$ and averaged $2.79 \pm 0.91\%$, i.e., about three out of 100 fish would be misclassified.

Of the total number of eggs used in each trial, on average $65.0 \pm 9.8\%$ were viable eggs. Mean survival up to 1 day after hatching of these viable eggs was $94.6 \pm 5.4\%$ in the untreated controls. Typically, one or two out of three trials were successful ($> 50\%$ survival 24 h AF in the untreated control). Survival at 1 day after hatching of treated groups relative to that of the untreated control is shown in Fig. 3. Survival decreased with longer shock durations, ranging from 82–97% (5 min shocks) to 61–71% (40 min shocks). Although there was a progressive decline in survival with longer shock durations, ANOVA did not detect significant differences when all groups were com-

Table 1
Analysis of variance of survival at 1 day post hatch in relation to treatment. In each trial, a total of 15 groups were used: one untreated control, two ''sham'' controls, and 12 cold-shocked groups (see Section 2 and Fig. 3). Analysis performed with the mean of three separate trials

Source of variation	Sum of squares	Degrees of freedom	Mean squares	*F*-ratio	Significance level
A. All 15 groups compared together					
Between groups	2342.59	14	167.32	1.059	0.4284
Within groups	4739.41	30	157.98		
B. ''Sham'' control for 10 min vs. cold-shocked groups for 10 min					
Between groups	395.96	3	131.98	1.653	0.2531
Within groups	638.78	8	79.84		
C. ''Sham'' control for 40 min vs. cold-shocked groups for 40 min					
Between groups	532.83	3	177.61	1.075	0.4127
Within groups	1321.56	8	165.19		

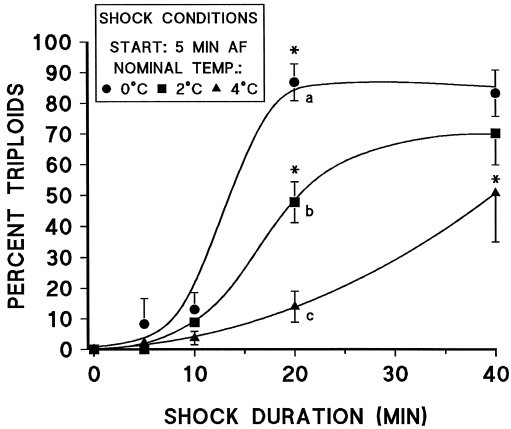

Fig. 4. Relationship between shock temperature and shock duration with the percentage of 3n turbot obtained. Shocks of 0–4°C were applied during 5–40 min, starting 5 min AF. Letters indicate significant differences ($P < 0.001$) between different shock temperatures with a shock duration of 20 min (the differences in the percentage of 3n turbot obtained between different shock temperatures were not significantly different at other shock durations). Within each shock temperature, asterisks indicate significant differences ($P < 0.001$) when compared to previous shock durations. Data as mean ± SEM of quadruplicate determinations per treatment from two separate trials.

pared together ($P > 0.05$; Table 1, A). In addition, survival of cold-shocked groups for 10 and 40 min was not significantly different ($P > 0.05$; Table 1, B and C) from the survival of their appropriate sham controls.

The number of triploid turbot larvae obtained with the different cold shocks assayed is shown in Fig. 4. Percent triploidy increased with both longer shock durations and lower shock temperatures. At 0°C or 2°C and at 4°C, shocks of 20 min or longer and 40 min, respectively, gave a significantly (ANOVA; $P < 0.001$) higher number of triploid turbot when compared to shocks of lesser duration at the same temperature. Maximum differences in triploid production as a function of temperature were evidenced with shocks of 20 min (ANOVA; $P < 0.001$). With these shocks, statistical analysis revealed a clear inverse relationship between shock temperature and number of triploid turbot obtained (Linear regression; $P < 0.001$; $r = -0.94$). Thus, with shocks of 20 min, the percentage of 3n turbot obtained was $78.6 + (-14.8$ times the shock temperature used in °C).

4. Discussion

This paper validates the use of NOR analysis for the determination of ploidy levels in the turbot and shows the effects of cold shocks on both triploidy rates and early larval survival in this economically important species. The highest triploidy rate, 87% (survival of 77.5% with respect to the untreated controls), was achieved with a cold shock of 0°C applied for 20 min. This represents a triploid yield of 67.3% and it compares well with

the triploid yield obtained in other species: olive flounder, 24.3% (Kim et al., 1993); halibut, 46–77.9% (Holmefjord and Refstie, 1997).

The chromosome number of control groups was in accordance with the chromosome number previously determined for this species (Bouza et al., 1994). The analysis of the number of nucleoli per nucleus is a straightforward and inexpensive technique to determine the level of ploidy. This technique has been applied when developing methods to induce triploidy in fish (Phillips et al., 1989; Felip et al., 1997), but can only be used when NOR are located in a single chromosome pair which results in the presence of one or two nucleoli per cell in diploids, and one, two, or three nucleoli in triploids. However, multichromosomal distribution of NOR has been observed in several vertebrate species (Arnheim et al., 1982; Volleth, 1987) including fish (Phillips et al., 1989; Castro et al., 1996). This can present difficulties due to the variable expression of available NOR in each cell. The turbot is a marine flatfish with a single NOR located in the short arm of each one of the only submetacentric pair of chromosomes (Bouza et al., 1994). However, a polymorphism in the number of NORs has been recently discovered in this species, although it affects only a small number of individuals (Pardo et al., 1998). The polymorphism manifests itself in that occasionally two different acrocentric chromosomes evidence Ag- and CMA_3-positive signals in their telomeres. Therefore, this fact could introduce some disturbance in evaluating the ploidy level when using the counting of nucleoli per cell in turbot. In the present work, we determined the number of NOR and verified the level of ploidy by karyotyping 20% of the individuals analyzed. The results obtained are in accordance with the previous data of NOR polymorphism in turbot (Pardo et al., 1998). Thus, diploid cells have, in some individuals in the same family, three nucleoli, while triploids exhibit four nucleoli, supporting the existence of additional active NOR. These individuals represent the upper tail of the mean nucleoli per cell distributions in diploids and triploids, respectively. This circumstance, however, only affected a minor fraction of the individuals analyzed, as expected, and diploid and triploid nucleoli number per cell distributions hardly overlapped. Checking the level of ploidy by karyotype analysis showed that the error committed by misclassification of individuals according to its level of ploidy is below 5%. Therefore, despite its NOR polymorphism, NOR analysis can be used to identify ploidy in the turbot.

Both untreated and sham controls were used in this study, the latter groups included to detect the effects of possible mechanical disturbances shortly AF due to handling. It was found that the survival of cold-shocked groups that gave high triploid ratios was not significantly different from that of appropriate sham controls. Thus, decreased survival from fertilization up to 1 day after hatching in the treated groups was probably a consequence of the handling involved in triploid induction, not of the triploidy condition per se. Regarding the actual survival values, here it is worth noting that survival, 1 day after hatching, was near 80% of the untreated controls, which is in accordance with that found in other studies on triploidy induction in marine fish (Felip et al., 1997), even though cultured turbot have typically lower larval survival (Devauchelle et al., 1988).

The timing of the cold shock and the temperature applied are in agreement with those used with other marine fish, i.e., commencement of shock within the first 15 min AF and shock temperature near or at 0°C. Regarding the best shock duration, our data show that shocks longer than 20 min did not increase the number of triploid larvae obtained.

This contrasts with the data obtained in other flatfishes where shock duration typically ranges between 45 min and 3 h (Lincoln, 1981; Tabata, 1991; Kim et al., 1993; Holmefjord and Refstie, 1997) and instead, is more similar to the short shocks (10 min) necessary to induce triploidy in the sea bass (Felip et al., 1997). It could be argued that a peak in the number of triploids obtained could exist at a shock duration between 20 and 40 min which was not tested. However, this is unlikely because for a given shock temperature and timing, once a maximum of triploidy is achieved, longer durations do not usually increase the number of triploids obtained as evidenced also in this study. The observation that shocks of more than 20 min did not increase the number of triploids obtained could be due to the warmer incubation temperatures used in the present study with turbot (and also in the study with sea bass) as compared with the relatively cooler temperatures used with other flatfishes. In fact, Díaz et al. (1993) suggested that the difference between the pre-shock and shock temperatures was more important than the shock temperature itself in obtaining high triploid rates. Thus, since sea bass and turbot eggs are incubated at 12–14°C before the shock in contrast to 6°C in halibut (Holmefjord and Refstie, 1997) or 7°C in plaice (Lincoln, 1981), it is likely that shocks near or at 0°C need to be applied for relatively shorter periods of time when compared to the cold water species referred to above.

This paper provides the basis for triploidy induction in the turbot by cold shocks AF. Although survival after the manipulations was acceptable, no 100% triploidy was achieved. In this regard, the inverse relationship between shock temperature and triploidy induction, i.e., $\%3n = 78.6 + (-14.8 \times °C)$ ($P < 0.001$; $r = -0.94$) derived from these trials predicts that $\sim$100% triploid turbot would be obtained with shocks of 20 min at -1.4°C, starting 5 min AF. This, however, is likely to negatively affect survival and thus other factors also important to ploidy induction, such as the time of commencement of the shock, should be taken into account. Optimization of treatment regimes to achieve 100% triploid turbot while maintaining high survival and the scaling up of these methods to enable the mass production of triploid turbot are currently being investigated.

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Artículo 2

Induction of triploidy in the turbot (*Scophthalmus maximus*). II. Effects of cold shock timing and induction of triploidy in a large volume of eggs

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Induction of triploidy in the turbot (*Scophthalmus maximus*) II. Effects of cold shock timing and induction of triploidy in a large volume of eggs

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Abstract

Triploidy was induced in the turbot (*Scophthalmus maximus*, L.) by applying cold shocks shortly after fertilization. The combined effects of the timing of cold shock commencement after fertilization, cold shock duration and cold shock temperature were investigated. Ploidy was assessed by counting the number of nucleoli per nucleus (NOR) in larvae and also by measuring erythrocyte size in juveniles. A clear peak in triploidy induction was obtained when shocks were started between 6 and 7 min after fertilization at a pre-shock temperature of 13–14 °C. With this timing, shocks of 20-min duration at 0 °C gave >90% triploidy, with survival about 80% of the untreated controls. In order to ensure both high triploidy rates and high survival, it was necessary to carefully maintain the water temperature just below 0 °C. Experiments with small and large volumes of eggs were performed in order to determine how changes in the relative volumes of eggs and chilled water could affect survival and triploidy induction. The best combination to induce triploidy in the turbot was as follows: shock commencement 6.5 min after fertilization, shock duration 25 min, and shock temperature between 0 and −1 °C. With this combination, 100% triploidy could consistently be induced with survival 60% of the untreated control. This was successfully applied to a large volume of eggs (~ 300 ml; 1 ml ~ 800 eggs) in order to mass-produce triploid turbot. Triploids had lower survival rate than diploids at hatching but similar thereafter, with the ability to complete the

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different stages of larval rearing, indicating the viability to produce triploid turbot under farming conditions.

Keywords: Sex control; Triploidy; Cold shocks; Turbot; *Scophthalmus maximus*

1. Introduction

Under culture conditions, turbot males grow significantly slower, do not reach as large size and mature earlier than females. Maturation, in turn, results in diminished growth, higher mortality and higher susceptibility to diseases (Imsland et al., 1997). Thus, there is interest in producing sterile or all-female stocks of turbot. This can be achieved by applying endocrine (Piferrer, 2001) or chromosome set manipulation techniques (Thorgaard, 1983; Felip et al., 2001), including the production of triploid and gynogenetic fish. Recently, the basis for triploidy induction in turbot was established using a small number of eggs and applying cold shocks shortly after fertilization (Piferrer et al., 2000). It was shown that a cold shock of 20-min duration at 0 °C resulted in 87% triploidy, with a survival rate 1 day after hatching of 77.5% relative to the untreated controls.

When developing triploidization treatments in fish, particularly in marine species, survival on a long-term basis is important, but survival at early stages of development is less relevant due to the usual high fecundity of these species. Instead, high triploidy rates are more important. Thus, when developing new protocols, it is better to first find the treatments that ensure 100% triploidy and then select those that, in addition, result in the highest possible survival. One hundred percent triploidy was not achieved in our previous study in turbot (Piferrer et al., 2000). Our interpretation was that other treatment variables such as shock timing should be improved, although the time selected, 5 min after fertilization (AF), was within the range (3–15 min AF) found to be appropriate for other flatfish species (Purdom, 1972; Lincoln, 1981; Tabata, 1991; Kim et al., 1993; Holmefjord and Refstie, 1997). In this regard, in a study with the sea bass (*Dicentrarchus labrax*), Felip et al. (1997) showed that of the three most usually investigated variables related to triploidy induction in fish, i.e., treatment timing, shock intensity and shock duration, treatment timing was the most important one, since it contributed more than 40% to the total variability associated with triploidy yield. Thus, even small changes in treatment timing may result in significant changes in the number of triploids obtained.

Felip et al. (1997) also found that the temperature of the shock was the second most important variable in terms of triploidy rates obtained. In our previous study with turbot, it was observed that shocks applied 5 min AF for 20 min at either 4, 2 or 0 °C (pre-shock temperature of 13–14 °C), resulted in 14%, 48% and 87% triploidy, respectively, while shocks longer than 20 min barely increased the number of triploids. Thus, a significant ($P < 0.001$, $r = -0.94$) inverse linear relationship between shock temperature and triploidy rates was demonstrated for a shock duration of 20 min (Piferrer et al., 2000). This relationship predicted that 100% triploidy could be obtained with a cold shock temperature of approximately −1 °C. However, it is likely that sub-zero temperatures would have a

negative impact on survival. In addition, management of a water bath near or at sub-zero temperature may introduce further variability to the treatment conditions for triploidy induction, especially when dealing with large volumes of eggs, as would be done under farming practice.

The objectives of the present study, therefore, were as follows: (1) to investigate the effects of timing of cold shock on survival and triploidy rates in the turbot in order to obtain 100% triploidy combined with the best possible survival; (2) to investigate the effects of shocks at sub-zero temperatures; (3) to attempt to scale-up the method for the mass-production of triploid turbot using the best combination of temperature, timing and duration of the cold shock; and (4) to study the early survival and growth of the resulting triploid turbot in comparison to their diploid counterparts.

2. Materials and methods

2.1. Gamete collection and artificial fertilization

Turbot broodstock were maintained under a constant photoperiod of 16-h light/8-h darkness and a water temperature of 13–14 °C during 60 days prior to maturation. For each repetition of each experiment (see below), eggs from one to two ovulated females (~ 1.1 mm diameter; 1 ml of eggs ~ 800 eggs), which were ready to be fertilized according to the criteria of McEvoy (1984), were obtained between April and June by abdominal massage and were coated with sperm from two to four running males (~ 200 μl of sperm for each 10 ml of eggs). For fertilization, each volume of eggs plus sperm was mixed with 2 vol. of seawater. Thirty seconds AF, eggs were gently rinsed with excess seawater for 30 s and then divided into groups of approximately 500 each (~ 100 eggs/ml) and kept in clean 20-ml glass vials until use. For large-scale experiments, eggs were divided into two groups (control and shocked) and placed in 2-l glass beakers. In any case, no attempts were made to separate viable and nonviable eggs. However, excess stripped eggs were poured into a graduate cylinder filled with seawater and used to estimate the percent viability calculated from the fraction of floating eggs.

2.2. Cold shock application and experimental design

For cold shocks, eggs were poured into glass vials or beakers containing water pre-chilled to the desired temperature and placed in a plastic tray filled with a mixture of crushed ice and seawater. Thus, the dilution for small-scale experiments (experiment 1) was 1:2, whereas for large-scale experiments (experiments 2 and 3) was 1:5. Actual water temperature inside the vials or beakers fluctuated by ± 0.5 °C as determined with a precision thermometer.

Experiment 1 was designed to determine the best shock timing. Cold shocks started either at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 or 20 min AF, and were applied at a nominal temperature of 0 °C (actual temperature of -0.7 ± 0.3 °C) for 20 min. In addition, three control groups were used. The untreated control consisted of fertilized eggs immediately transferred to the incubators (see details below) with no further disturbance. This group

served as control for gamete quality and artificial fertilization procedures. The other two remaining control groups were sham controls. These were composed of eggs subjected to the same manipulations applied to cold shocked eggs (pouring into glass vials and handling), but they were always maintained at 13–14 °C. These controls served to evaluate the effects of possible mechanical disturbances. One sham control group was transferred to the incubators 10 min AF and the other 20 min AF, i.e., at times corresponding approximately to 1/3 and 2/3 of the total duration (28 min) of the cold shock treatments, from the first to the last group to be treated.

Experiment 2 was designed to study triploidy induction in large volumes of eggs (150–300 ml) evaluating, at the same time, the effects of sub-zero temperatures. Shocks were applied 5 min AF for 20 min at an actual temperature of -1.4 ± 0.1 °C (first trial) or 6.5 AF for 25 min at 0.4 ± 0.1 °C (second trial) or -0.1 ± 0.0 °C (third trial). The ranges indicate variation within trials (i.e., the range of temperatures from start to finish of the cold shock). Cumulative shock temperature was obtained by multiplying mean actual shock temperature by shock duration (°C × min).

Experiment 3 was carried out using the combination of the best shock conditions as determined above on large volumes of eggs (200–400 ml) to assess the early viability and growth of mass-produced triploid turbot. Shocks were applied 6.5 min AF for 25 min with temperatures between -0.5 and -1.2 °C. In all experiments, control and treated groups were incubated at 13–14 °C in a recirculated water system as described previously (Piferrer et al., 2000).

2.3. Calculation of survival and ploidy determination

Hatching typically took place over 1 day, 5 days AF. The non-fertilized eggs, non-hatched eggs and larvae were counted and added to obtain the total number of eggs initially used in each group. Survival was calculated 1 day post hatch (DPH) as the number of larvae alive with respect to the number of initial eggs and was expressed as a percentage.

Ploidy was determined in larvae collected 1 DPH by counting the number of nucleoli per nucleus in 50 cells from each larva ($n = 32$ larvae per replicate). Cold shocked larvae with mean number of nucleoli >1.735 were considered triploids (Piferrer et al., 2000). In juveniles (6–9 months of age), ploidy was also determined by measuring the large axis of erythrocytes ($n = 20$–40 erythrocytes/fish; ~ 260 fish per ploidy).

2.4. Determination of weight and length of larvae

Because of the small size of turbot larvae right after hatching and in order to avoid errors due to the water attached to them by capillarity, larvae were dried before weighing. Ten larvae were dried for 24 h at 65 °C and weighed to 0.1 mg in a precision balance. Measured length was the total length.

2.5. Statistical analysis of data

Each control and treated group was carried out in quadruplicate (experiment 1), triplicate (experiment 2) or duplicate (experiment 3). Each experiment was repeated at

least three times with eggs from different females to account for possible variability between fish-to-fish. Survival at 1 day after hatching was transformed to percentages and expressed relative to the survival of the untreated control (Felip et al., 1997; Piferrer et al., 2000). Only those experiments in which actual survival 1 DPH was above 50% in the untreated controls were used. Percentage data were arcsine transformed before analysis of variance (ANOVA). Differences in growth between ploidies were assessed with Student's *t*-test. Data are expressed as mean ± S.E.M.

3. Results

To accurately determine the most appropriate time AF to start the cold shock, large differences in triploid turbot production needed to be elicited with small variations in shock timing. Thus, in experiment 1, shocks were deliberately applied for the minimum effective duration (20 min) and at sub-zero temperatures, as previously determined (Piferrer et al., 2000). A peak of triploid production was observed when the shock started between 5 and 7 min AF, with the number of triploids obtained significantly higher (ANOVA, $P<0.05$) than those obtained with shocks applied at earlier or later times (Fig. 1). Results also revealed that >90% triploids were obtained with shocks starting 6–7 min AF, but that a 20-min duration was not sufficient to ensure 100% triploidy.

The number of viable eggs averaged 65.0 ± 9.8% of the total number of eggs used. Mean survival up to 1 DPH of these viable eggs was 94.6 ± 5.4% in the untreated controls. Survival at 1 DPH of treated groups relative to that of the untreated control is shown in Fig. 2. Survival was below 50% ($P<0.05$) when the cold shock was

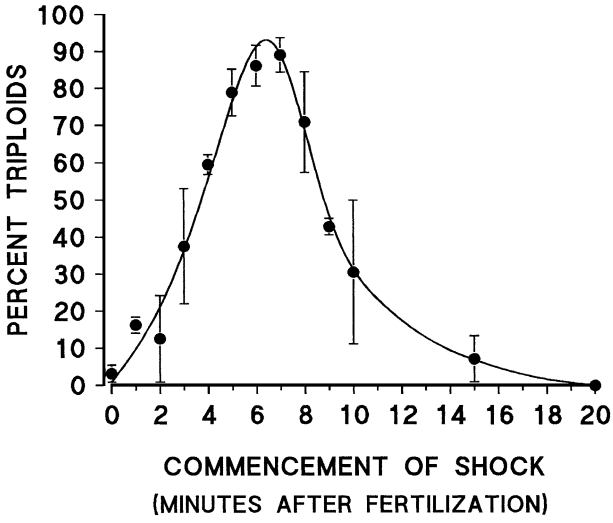

Fig. 1. Relationship between shock timing and percentage of triploid turbot obtained. Shocks of −0.7 ± 0.3 °C were applied during 20 min, starting from 0 to 20 min after fertilization. Data are expressed as mean ± S.E.M. of quadruplicate determinations per treatment from four separate experiments.

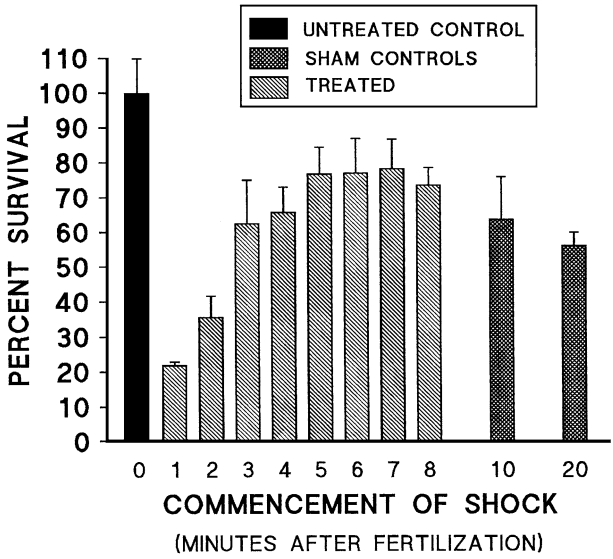

Fig. 2. Relationship between shock timing and survival at 1 day after hatching. Shocks of -0.7 ± 0.3 °C were applied during 20 min, starting from 0 to 20 min after fertilization. Survival is expressed relative to untreated controls. See Materials and methods for a detailed explanation of the different types of controls used. Data expressed as mean ± S.E.M. of quadruplicate determinations from three separate experiments. Survival of treated groups at 9, 10, 15 and 20 min AF was ~ 70% but was measured only in one experiment and thus is not graphed.

applied 1 or 2 min AF, then increased until it reached ~ 78% with shocks starting at 5 min AF and had similar values with shocks starting at 6 or 7 min AF (not significantly different from sham controls at 10 and 20 min). Thus. maximum survival was recorded with shocks starting between 5 and 7 min AF. Survival decreased to ~ 60% with shocks starting at 20 min AF.

The combined influence of timing, shock intensity and duration when applied to a large number of eggs was investigated in experiment 2. This experiment (Table 1) verified our prediction that 100% triploidy could be obtained with sub-zero temperatures. However, low temperatures negatively affected survival (first trial). A decrease (~ 10%) in survival was observed when water temperature during the shock was maintained below 0 °C (third vs. second trial). Thus, the cumulative temperature during the shock (°C × min) was relevant on survival. Triploidy yield was close to 60% 1 day after hatching in the last two trials. The evolution of water temperatures inside the glass vials or beakers, where the eggs were maintained before and also after the shocks started, is shown in Fig. 3. This figure illustrates the situation in laboratory-scale experiments, regardless of success (as in experiment 1), vs. successful mass-productions (as in the third trial of experiment 2), when large volumes of pre-chilled water were used to cold shock a relatively large volume of eggs. Thus, for successful induction of 100% triploidy, especially when dealing with a large volume of eggs, the water temperature during the first 5 min after the commencement of the shock should not exceed 0 °C (Fig. 3).

Table 1
Summary of the results obtained from experiments carried out with large amounts of eggs, in which the predicted suitable combinations of the three shock variables studied (timing, duration and temperature) were used for mass-production of triploid turbot

	First trial	Second trial	Third trial
Volume of eggs used (ml)	150	300	285
Approximate number of eggs ($\times 10^3$)	118.3	236.6	224.8
Shock start (min AF)	5	6.5	6.5
Shock duration (min)	20	25	25
Shock temperature (°C)	-1.4 ± 0.1	0.4 ± 0.1	-0.1 ± 0.0
Cumulative temperature (°C × min)	-27.2 ± 0.3	10.3 ± 1.4	-2.1 ± 1.1
Survival (% of controls)	18 ± 1	71 ± 1	59 ± 7
Triploidy (%)	92 ± 2	83 ± 3	100 ± 0
Triploid yield (%)	17	59	59

AF: after fertilization. Temperature and survival data are expressed as mean ± S.E.M. of triplicate determinations in each trial.

The evolution of weight and length of triploid turbot larvae when compared to diploids is shown in Fig. 4. No differences were detected ($P>0.05$) between the two ploidies in any of the two growth variables measured. The actual ploidy level was verified in a sample of treated larvae and in juveniles (6–9 months of age). Data showed that, in the mass-production of experiment 3, the eggs that were cold shocked resulted in 95–100% triploids, depending on the trial, as assessed by counting the number of nucleoli per nucleus and verified by measuring the large axis of

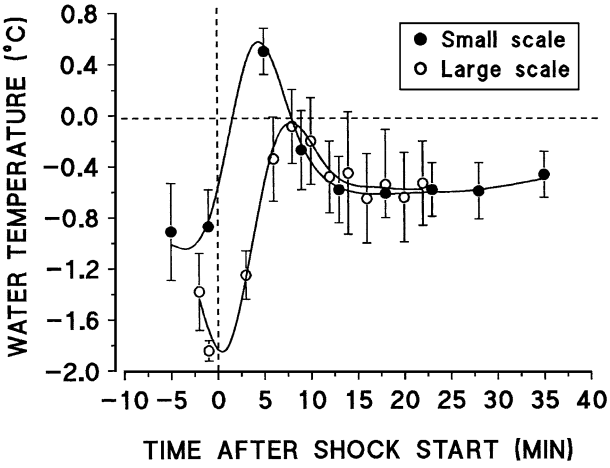

Fig. 3. Actual temperature of the water inside the glass bottles or beakers just prior (left of the vertical dashed line) and during (right of the vertical dashed line) the time the nominal shock temperature was set at 0 °C. At time 0, 1 vol. of eggs in water at 13–14 °C was added to ~2 vol. (small-scale experiments) or ~5 vol. (large scale experiments) of pre-chilled water and the cold shock started. Note that only in the small-scale experiments were eggs briefly exposed to water at >0 °C (above the horizontal dashed line). This did not occur in the large-scale experiments due to the larger dilution. Data expressed as mean ± S.E.M. of quadruplicate determinations from four separate experiments.

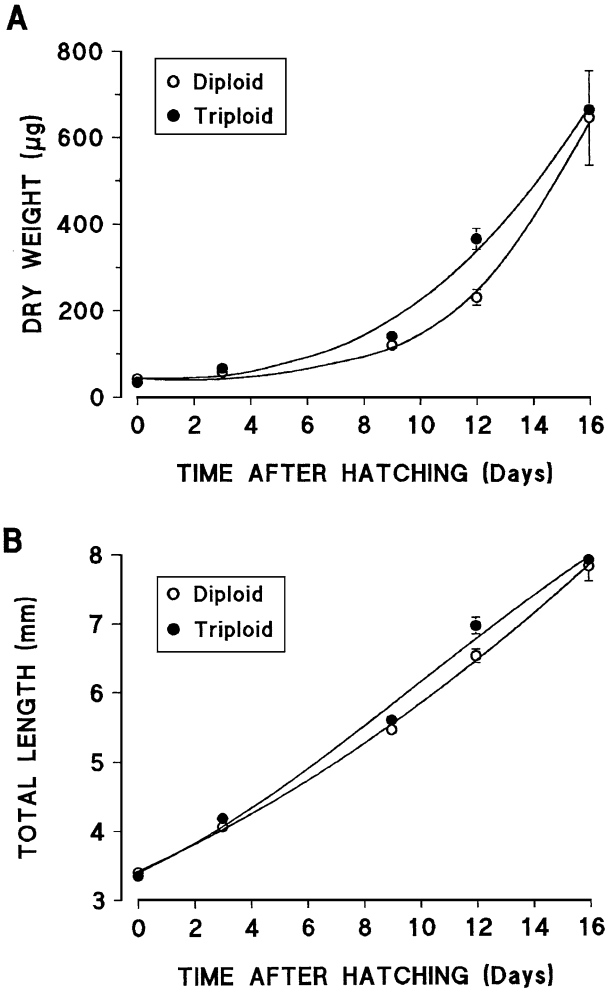

Fig. 4. Comparative growth in weight (A) and length (B) between diploid and triploid turbot larvae. Data expressed as mean ± S.E.M. of 10 determinations per ploidy from two separate experiments. S.E.M. values equal or smaller than the datapoint symbols are not plotted.

erythrocytes. In diploids ($n = 240$ fish), the length of this axis was 10.36 ± 0.05 μm whereas in triploids ($n = 280$ fish) it was 13.82 ± 0.06 μm ($P < 0.001$). The growth and survival of triploid turbot at 1 year of age and thereafter will be reported elsewhere.

4. Discussion

A method to mass-produce triploid turbot was developed which involved applying cold shocks shortly after fertilization, and was scaled-up to treat large volumes of turbot eggs.

The highest triploidy rate, 100% (survival of about 60% with respect to the untreated controls), was achieved with cold shocks just below 0 °C applied for 25 min, starting 6.5 min after fertilization. The turbot is an economically important species for aquaculture in Europe. Turbot females outgrow and mature later than males (Imsland et al., 1997). The methodology developed here will enable the mass-production of triploid turbot, which presumably will remain sterile, although this needs to be confirmed. Furthermore, when combined with the use of irradiated sperm, these cold shocks have resulted in the production of gynogenetic turbot (Piferrer et al., unpublished observations). If further analysis shows that this species exhibits female homogamety, the production of gynogenetics can be a means to obtain all-female progeny, either directly (Howell et al., 1995) or in combination with the method of indirect feminization (Piferrer, 2001).

When compared with the most appropriate conditions established for other marine teleost species including flatfish (Felip et al., 2001), the conditions established for turbot are similar in terms of timing and temperature of the shock but differ in its duration. This is probably due to the fact that other flatfishes for which cold shocks have been tested are cultured at 6–7 °C, as is the case of plaice and halibut (Lincoln, 1981; Holmefjord and Refstie, 1997), in contrast to the 12–14 °C customary of turbot culture. In this regard, it is a well-known fact that the difference between the pre-shock temperature and the shock temperature is more important than the actual temperature of the shock itself (Díaz et al., 1993).

The relationship between shock temperature and survival was analyzed in this study in two different scenarios: in small- or laboratory-scale experiments, as is customary when developing methods for chromosome set manipulation in fish, and in large-scale experiments, in order to reproduce the actual conditions that this methodology would face when applied in production facilities. To the best of our knowledge, this is the first time that the temperature of the water containing the eggs was recorded before and during the cold shock and related to both the dilution (1:2 or 1:5) in a specific volume of pre-chilled water and the resulting triploidy and survival rates. By using ‘‘cumulative temperature’’, to integrate the variations of the water during the duration of the shock, it became apparent that when dealing with a large number of eggs (>200 ml), it is important to maintain an appropriate amount of pre-chilled water at around −2 °C in such a way that when the eggs are poured at the onset of the shock, the resulting mixture does not surpass 0 °C, particularly during the first 5 min. Nevertheless, the volumes of eggs and pre-chilled water should be adjusted in such a way that after the first 5 min, the temperature of the mixture is ideally maintained at around −0.5 °C but not lower. This will ensure a triploidization rate of 100%, or very close to it, while survival of the shocked eggs is maintained around 60–70% of the survival of the unshocked control.

In regard to the actual survival values, here it is worth noting that survival 1 DPH was near 80% in small-scale experiments, which represents the upper range of survival at this age when compared to that found in other studies involving triploidy induction in marine fish (Felip et al., 2001). However, cultured turbot typically have a lower larval survival (Devauchelle et al., 1988). The consequences of using large volumes of eggs in large-scale trials is that survival is further reduced to 60–70% of the controls, even if trials are optimized for all three major shock variables (timing, duration and temperature). The reason for this further decrease in survival is not known but could be related to increased

mechanical disturbance when handling large amounts of eggs. Likewise, another observation for which we have no clear explanation is the significantly lower survival of eggs observed when the cold shock was administered just 1 or 2 min AF (Fig. 2). Because the triploidy rates are maximal when shocks are started between 4 and 7 min AF, this suggests that the extrusion of the second polar body starts during this period in fertilized turbot eggs maintained at 12–14 °C. Thus, the lower survival with shocks applied 1 or 2 min AF could reflect interference or disturbances with events occurring in the cytoplasm of the egg and related to the resumption of meiosis in preparation for the extrusion of the second polar body.

In summary, the optimal conditions to induce triploidy in turbot by cold shocks are the following: time after fertilization when the shock is started (for broodstock cultured at 13–14 °C), 6.5 min; duration of shock, 25 min; water temperature during the shock: −1 to 0 °C. Thus, a large number of turbot eggs (likely to be used in turbot hatcheries), e.g., one-quarter million eggs (~ 300 ml) can be made triploid by placing them as indicated above in water chilled at about −2 °C. It is very important that the temperature of the water where eggs are kept is maintained below 0 °C, especially during the first 5 min of the shock. Also, for easier operation, it may be desirable to split the ~ 300 ml of eggs in aliquots of smaller volume to facilitate handling. With this method, routinely 95–100% triploid turbot larvae are obtained with a survival of about 60% of the diploids. The data available so far also indicate that there are no significant differences in growth between diploids and triploids at an early age. The growth and gonadal development of mass-produced triploid turbot is currently being studied and will be reported elsewhere.

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Artículo 3

Growth and Gonadal Development in Diploid and Triploid Turbot (*Scophthalmus maximus*)

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Growth and gonadal development in diploid and triploid turbot (*Scophthalmus maximus*)

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Abstract

This study determined the effect of triploidy on the survival, growth and gonadal development of turbot from 6 to 48 months of age. From 6 to 24 months of age (first sexual maturity), survival was similar in both ploidies ($P>0.05$). From 24 to 48 months of age, after the first sexual maturity, survival was 91.9% in diploids and 100% in triploids, which did not exhibit the post-spawning-associated mortality. Growth was similar for both ploidies during the first year of life. After that, triploids grew significantly ($P<0.05$) more that diploids, with more marked differences after each spawning season. From 24 to 48 months, the average weight difference between both ploidies was $11.4 \pm 1.9\%$, ranging from 4.3 to 23.0%. At 47 months of age, the biomass of triploids was 10.3% greater in total weight and 14.3% greater in eviscerated weight. Gonads of triploid males were similar to that of diploids, whereas in triploid females, they were significantly smaller and rudimentary. A histological analysis carried out at 47 months of age showed complete sterility of triploids in both sexes. Sex ratio was 1 male (M):0.6 female (F), for diploids, significantly ($P<0.05$) different from 1:1, and 1 M:3.3 F for triploids, significantly ($P<0.05$) different from 1:1 and from the diploids. Since females grow more than males, culture of triploids benefited from the high female ratio, which helped to reduce size dispersion. In addition, their sterility allowed better performance by avoiding the reduction in growth that takes place during the spawning periods. Together, these observations indicate that triploidy induction can be an interesting option for turbot aquaculture, especially for the production of large-size fish of more than 2 years of age.

Keywords: Triploidy; Growth; Gonadal Development; Turbot (*Scophthalmus maximus*); Sex ratio

1. Introduction

Turbot (*Scophthalmus maximus* L.) is a marine teleost which has an important commercial value. It is essential for European aquaculture industry, especially in Galicia (NW of Spain), where it generates

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85.0% of the Spanish turbot production and 45.4% of the world turbot production (Ojeda, 2003). The growing phase starts using animals weighing 5–10 g which are 3–4 months old. Turbot is a fast growing species which reaches 1.5 kg at an age of 25–29 months. Although it can be marketed as soon as it reaches 0.5 kg, most fish are marketed in the range 1.5–4 kg, depending on the end user (restaurant or domestic consumption). Based on size, fish will be eaten as a whole (~ 0.5 kg) or after being filleted.

Under culture conditions, the first sexual maturity takes place at an age of about 24 months, during the growing period. As it is the case in many fish, the first maturity does not negatively affect growth, since by that time, the gonads are still not too big. Nevertheless, animals which are cultured until they reach 4 kg undergo a second and a third sexual maturity, with more marked effects on growth than in the first sexual maturity, due to their bigger gonads. Sexual maturation disturbs behavior and slows down growth, as fish reject food and are more sensitive to changes in water temperature and low oxygen levels, which translates into mortalities during and after these periods. Therefore, induced sterility could be an effective method to solve or alleviate these problems in the culture of big-sized turbot.

Triploidy induction is an effective way to achieve sterility in fish (Swarup, 1959; Chourrout, 1987; Carrasco et al., 1999; Felip et al., 2001a; Zanuy et al., 2001). Triploidy alters chromosome pairing during meiosis and thus gonadal development is impaired, especially in females, where meiosis occurs after a relatively short period of growth by mitosis. In males, gonadal development is similar to that of diploids, and some species are even capable of producing sperm, although it is very diluted and aneuploid (Benfey, 1999). Sterility confers a potential additional advantage, as the energy invested in reproduction can be diverted to somatic growth (Utter et al., 1983; Ihssen et al., 1990; Benfey, 1999). Triploidy, however, does not always result in larger body size (Felip et al., 2001b).

Triploidy induction by chromosome set manipulation has been successfully carried out in several marine species with commercial interest, such as plaice, *Pleuronectes platessa* (Purdom, 1972), Atlantic salmon, *Salmo salar* (Johnstone, 1985), gilthead sea bream, *Sparus aurata* (Garrido-Ramos et al., 1996), halibut, *Hipoglossus hipoglossus* (Holmefjord and Refstie, 1997), sea bass, *Dicentrarchus labrax* (Felip et al., 1997), and turbot, *Scophthalmus maximus* (Piferrer et al., 2000, 2003). However, many studies have focused on determining the combination of treatment variables to induce triploidy, with less attention being paid on the actual performance of triploids under culture conditions. In those studies in which this has been done, results have been equivocal. Thus, in adult *Platichthys flesus*, triploids grew more than diploids (Lincoln, 1981), in *Paralichthys olivaceus* and *Pagrus major*, triploids grew less (Arai, 2001), and in *Dicentrarchus labrax*, triploids grew equally in the case of juveniles and less in the case of adults (Felip et al., 1999, 2001b). Based on the above, the aim of this study was to determine the survival, growth and gonadal development of triploid turbot during 4 consecutive years.

2. Material and methods

2.1. Animals used

The fish used in this experiment were reared at the Instituto Español de Oceanografía in Vigo (Spain). Eggs, all them from the same batch and from one female, and a pool of sperm from two males were obtained from fish of the same broodstock, in June 1999. Triploidy was induced by applying a cold-shock shortly after fertilization according to Piferrer et al. (2000, 2003). The ploidy level was verified in 6-month-old juveniles by determining the size of the erythrocyte major axis (20–40 erythrocytes per fish, $n=240$ diploids and $n=280$ triploids) in a blood sample, stained with Hemacolor (E. Merck. Darmastadt., Germany) as previously described (Piferrer et al., 2003). Mean erythrocyte major axis length ranged between 10.0 ± 0.21 μm and 11.4 ± 0.21 μm in control diploids and between 13.0 ± 0.16 μm and 15.0 ± 0.26 μm in cold-shocked fish ($P<0.001$). Thus, the fish used in the "triploid" groups were in fact 100% triploids.

Two hundred 6-month-old control diploids and two hundred verified triploids of the same age were placed in four 3800-l tanks. Each tank contained 100 fish, two of them with diploids and two with triploids, all with the same initial mean weight and similar weight

frequency distributions. Tanks were provided with an open water circuit, and fish were reared under natural conditions of photoperiod and temperature. Fish were fed by automatic feeders with dry pellets of increasing size (Trouw, Burgos, Spain) 7 days a week until they reached 24 months of age, and 3 days a week thereafter. When they were 2 months old, fish were vaccinated against *Vibrio* sp. by immersion with Gava-3 (Laboratorios HIPRA, Girona, Spain). Periodic formol baths were administered throughout the experiment to prevent the presence of parasites.

2.2. Experimental design

Each fish was weighted (precision 0.01 g) and measured (precision 0.1 cm) every 2 months from 6 to 42 months, and every 3 months from 42 to 48 months of age. At 8, 14, 20, 24, 26, 32 and 36 months of age, a total of 66 diploids and 70 triploids were sacrificed (~ 10 fish/ploidy/sampling). Length weight, sex, gonad weight were determined according to ploidy and sex.

A bacterial infection which took place in one of the diploid fish tanks at an age of 20 months caused the mortality of 10 out of the 70 remaining fish. In order to equalize fish densities and minimize the impact of this infection on the growth study, 7 fish were removed from the other diploid tank. Four of these fish were discarded and the other three were introduced in the tank that had suffered the infection. In addition, 7 fish were removed from each triploid tank. Furthermore, at 23 months, all remaining fish ($n=60$) from one of the triploid tanks died as a result of the failure of the support system. Therefore, all fish ($n=58$) from one diploid tank (no differences in weight and length between fish of the two diploid tanks) were removed from the experiment. The fish from the remaining unaffected two tanks – one triploid tank ($n=62$) and one diploid tank ($n=62$) – were maintained under the same rearing conditions until the end of the experiment, when they reached an age of 48 months.

At 47 months of age and just before spawning, 20 fish from each ploidy were sacrificed, and total weight and eviscerated weight per ploidy and sex were determined. The biomass of each tank was estimated taking into account the total or the eviscerated weight. Gonads were histologically analyzed and the sex ratio determined.

2.3. Morphometric parameters

Total length (cm) and average weight (g) were calculated during each sample and differences were statistically compared between both ploidies. The condition factor (K) was calculated as weight (W) divided by length (L) cubed (W/L^3). Specific growth rates for weight (SGRw) and length (SGR_L) were calculated for each period between samplings as ($SGR_W=100$ (Ln Wt − Ln Wi)/t and $SGR_L=100$ (Ln Lf − Ln Li)/t, where Wt and Lt are final weight and length, and Wi and Li are initial weight and length for the given period of t days.

2.4. Analysis of weight frequency distributions

Weight frequency distributions were determined during the spawning periods (April and June 2000, 2001, 2002 and March and June 2003) and the resting periods (December 1999, 2000, 2001 and January 2002). Distributions were compared using the Kolmogorov–Smirnov test. The coefficient of variation was calculated in each period to assess weight dispersion in the rearing tanks.

2.5. Body indices

The gonadsomatic index (GSI) was determined for all the sacrificed fish according to the formula: GSI = 100 (gonadal weight / total weight).

2.6. Gonadal histology and sex ratios

Gonadal development was determined in 47-month-old fish (20 of each ploidy). Gonads were fixed in 2.5% glutaraldehyde in 0.025 M cacodylate buffer (pH 7.4) and processed using conventional histological procedures. Sex ratios were macroscopically determined in 150 triploids and 144 diploids.

2.7. Statistical analyses

Data were expressed as a mean ± SEM. Statistical analyses were carried out using the statistical package SSPS, 11.5. (Imation Enterprises Corp. Oakdale, MN, U.S.A.). Comparison of erythrocyte size, fish weight and length between diploids and triploids was made using the t-Student test. Levene's test was used

to determine the homogeneity of variances. The Kruskall–Wallis test was used when distributions were not normal according to the Kolmogorov–Smirnov test. Frequency distributions of weight were compared using the test for two samples of Kolmogorov–Smirnov. Body indices were analyzed in each sampling using the Kruskall–Wallis test. The Chi-squared test (χ^2) was used to determine differences in sex ratios between ploidies. Differences were considered significant when $P<0.05$ (Sokal and Rohlf, 1981).

3. Results

Survival during the juvenile phase, from 6 to 24 months of age, was 87.0 ± 11.3% in diploids and 94.0 ± 1.4% in triploids ($P>0.05$). After the first sexual maturation (24 months), survival was 91.9% in diploids and 100% in triploids, indicating that the post-spawning period-associated mortalities did not take place in the triploids. No malformations were observed in any of the groups, neither in the jaw nor in the spine.

From 6 to 48 months of age, diploid fish grew in length from 15.7 ± 0.07 to 51.5 ± 0.73 cm, and in weight from 83.8 ± 0.94 to 2934.5 ± 133.74 g, while triploids grew in length from 15.9 ± 0.06 to 53.9 ± 0.50 cm and in weight from 83.6 ± 0.94 to 3608.2 ± 103.73 g (Fig. 1A–B). During the juvenile stage and until the first sexual maturation, growth in length and weight was similar between both ploidies, being the triploid's average length and weight usually greater than that of diploids, but differences were only significant in the period from 16 to 18 months of age ($P<0.05$). After 24 months of age (first sexual maturation), triploids had higher length than diploids until the end of the experiment. The weight of triploids was also greater than that of diploids, although differences were only significant after the spawning periods, when the diploids weight decreased due to gonadal regression. From an age of 26 to 48 months, the average weight difference between diploids and triploids was 11.4 ± 1.9%, ranging between 4.3 and 23.0%.

At an age of 47 months, immediately after the spawning period (third sexual maturation), total and eviscerated weight per sex was determined in a sample of 20 fish per ploidy. Total weight was 2412.5 ± 131.6 g for diploid males, and 2800.0 ± 174.6 g ($P>0.05$) for triploid males, whereas it was 4093.7 ± 162.7 g for diploid females and 3554.2 ± 186.4 g for triploid females ($P>0.05$). Eviscerated weight was 2237.7 ± 117.6 g for diploid males and

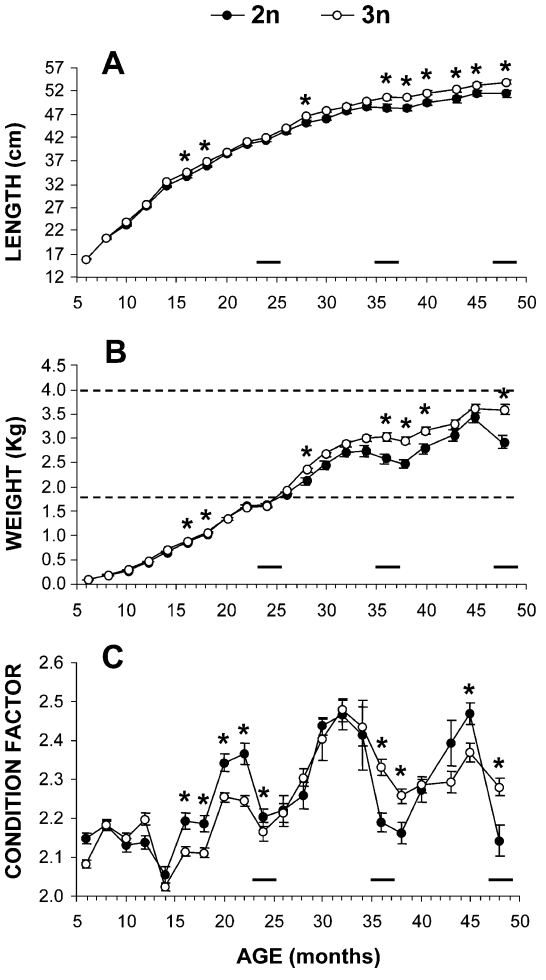

Fig. 1. Diploid and triploid turbot length (A), weight (B) and condition factor (C) from 6 to 48 months of age. Data from an age of 6 to 24 months are represented by the mean ± SEM of 2 diploid groups ($n=60$–100 fish per group) and 2 triploid groups ($n=61$–100 fish per group). Data from 26 to 36 months of age are represented by the mean ± SEM of 2 diploid groups ($n=22$–31 fish per group) and 2 triploid groups ($n=25$–31 fish per group). Data from 38 to 48 months of age are represented by the mean ± SEM of a diploid group ($n=25$–45 fish) and a triploid group ($n=30$–50 fish). Asterisks indicate significant differences ($P<0.05$) between ploidies. Black bars indicate the spawning periods. Dotted lines indicate the weight range currently used during turbot marketing.

2628.6 ± 165.1 g for triploid males ($P > 0.05$), while it was 3543.8 ± 139.0 g for diploid females and 3287.5 ± 167.6 g for triploid females ($P > 0.05$). The biomass was higher in triploids than in diploids, being this difference of 10.3% when taking into account the total weight or 14.3% when taking into account the eviscerated weight (Table 1).

Weight frequency distributions for fish of both ploidies are shown in Fig. 2A–L. The coefficient of variation (CV) in weight increased in the diploids after their first sexual maturation, reaching 30%, and reflecting an increase in the percentage of smaller fish. In triploids, the CV did not exceed 20%, indicating a more homogenous weight distribution.

The condition factor (K) during the first year was similar for both ploidies, and from 16 to 24 months of age was higher in diploids than in triploids ($P < 0.05$). After the second sexual maturation, the K of triploids was generally higher ($P < 0.05$) during the post-spawning periods and lower than that of diploids during the pre-spawning periods, reflecting the greater increase in weight of diploids during sexual maturation. These differences were more marked during the fourth year of life (third sexual maturation) (Fig. 1C).

Specific growth rates in length and weight were similar for both ploidies. Values regularly decreased as the individuals grew older from an age of 8 to 24 months. Since then, during the third and the fourth year of life, growth rates oscillated in a similar way in both ploidies, increasing and decreasing according to the reproductive cycle (Fig. 3).

Cumulative sex ratio (taking into account not only the sex of the fish at the final sampling but also the sex of fish sacrificed during periodic samplings where sex could be determined to assess overall sex ratio) was 61.3% males and 38.7% females (1 M:0.6 F) in diploids ($n = 144$) and 23.1% males and 76.9% females (1 M:3.3 F), in triploids ($n = 150$) with significant differences ($P < 0.05$) between ploidies and with respect to the 1:1 sex ratio.

The gonadsomatic index (GSI) of females according to ploidy was significantly different ($P < 0.05$) after an age of 10 months, reaching values of 13% in diploid females during the second and the third sexual maturation, and between 0.13–0.14% in triploid females during the same period. In males, differences were only significant at 32, 36 and 48 months of age ($P < 0.05$) (Fig. 4A–B).

Male and female diploid gonads were well developed, in accordance with fish age and season. Macroscopically, testes of triploids were similar to that of diploids, while ovaries of triploids were markedly smaller and had a rudimentary appearance (Fig. 5). In diploids, sperm was easily obtained from most males during the reproductive season. Likewise, most females matured, producing eggs usually during May and June of each year. In triploids, it was very difficult to externally distinguish males from females – just a smaller size in males was noticed, but not in every case – and no sperm was obtained even after abdominal massage. Females did not show any sign of sexual maturation. Histological analyses showed that diploid females had ovaries containing oocytes in different vitellogenic stages. Triploid females mostly had primary oocytes and oogonia, although oocytes in the perinucleolar stage and in the early stages of vitellogenesis were sporadically observed. Well-developed spermatozoa were observed in the testes of all diploid males,

Table 1
Total and eviscerated mean weight per ploidy and sex, and estimated biomass per ploidy at an age of 47 months

Ploidy	Sex	%	Total weight (g)	Total biomass (kg) per 100 fish	Eviscerated weight (g)	Total biomass (kg) per 100 fish
Diploids	Males	61.3	2412.5 ± 131.6	306.5	2237.7 ± 117.6	274.3
	Females	38.7	4093.7 ± 162.7		3543.8 ± 139.0	
Triploids	Males	23.1	2800.0 ± 174.6	338.0	2628.6 ± 165.1	313.5
	Females	76.9	3554.2 ± 186.4		3287.5 ± 167.6	

Each weight value is an average ± SEM ($n = 20$).
Differences in total weight or eviscerated weight at an age of 47 months were not significant, neither between males and females regardless of ploidy ($P > 0.05$).

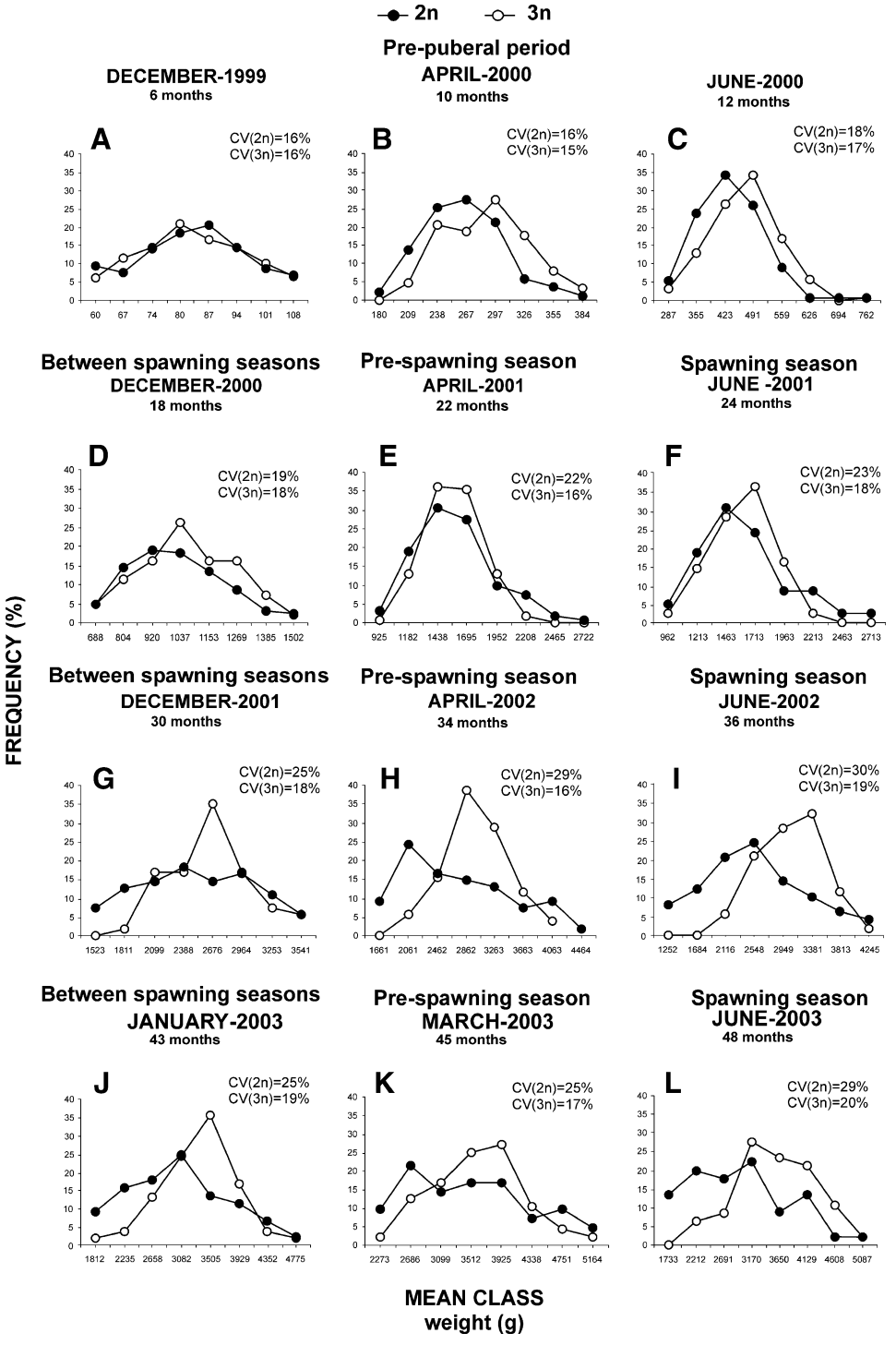

Fig. 2. Diploid and triploid weight frequency distributions from 6 to 48 months of age, according to the reproductive cycle. The variation coefficient (CV) is indicated for each period.

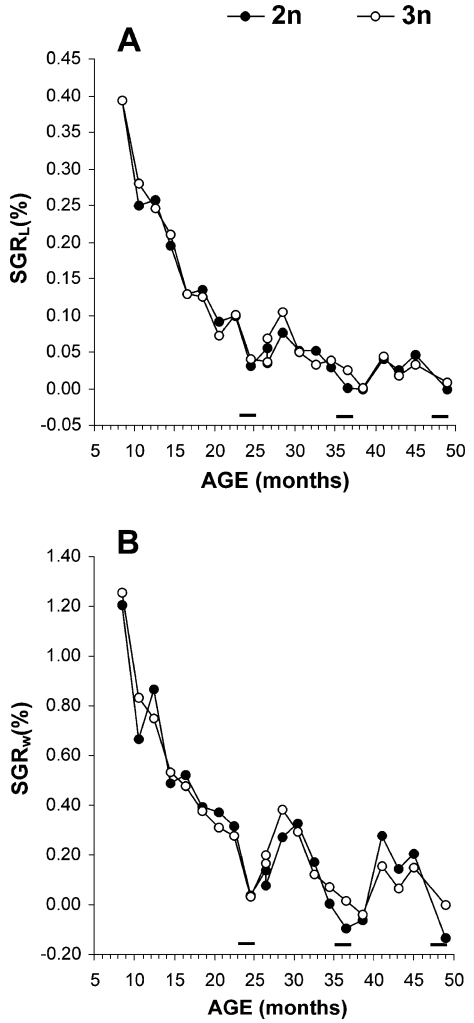

Fig. 3. Diploid and triploid turbot specific growth rate in length (A) and weight (B) from 8 to 48 months of age. Data from an age of 8 to 36 months are represented by the mean of two diploid groups and two triploid groups. Data from an age of 38 to 48 months are represented as the mean of a diploid group and a triploid group. Black bars indicate the spawning periods.

while in triploid males, no spermatozoa were observed in any fish.

4. Discussion

As previously shown in several fish species such as, for example, salmon (Benfey and Sutterling, 1984) or sea bass (Felip et al., 1997), we recently showed that > 90% triploid turbot can be mass-produced, with high larval survival and low cost, starting with a large volume of eggs (Piferrer et al., 2000, 2003). This demonstrated the feasibility of obtaining triploids in this economically important species under commercial conditions. Here, we extend our observations providing essential information on the performance of triploid turbot. This study also helps to alleviate the relatively small amount of information available on the effects of triploidy in flatfish. An important consideration is survival. During the juvenile stage, triploids had a survival similar to that of diploids. However, after the first sexual maturation, survival was higher in triploids, since females were not affect-

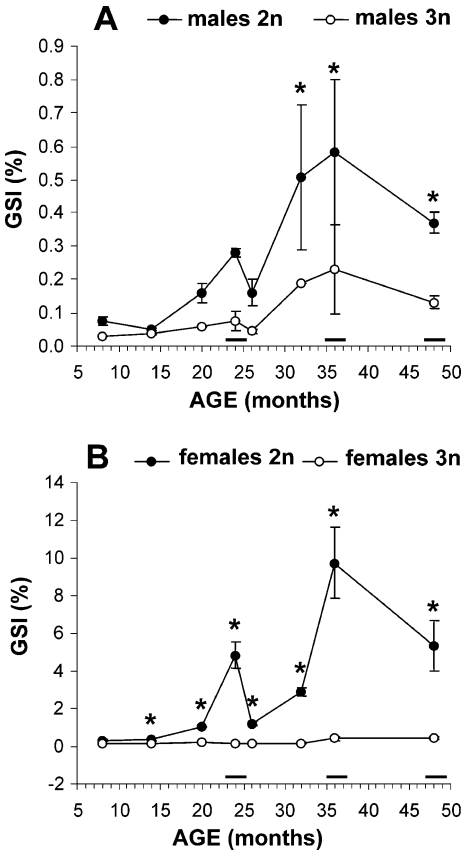

Fig. 4. Male (A) and female (B) diploid and triploid turbot gonadsomatic index from 8 to 48 months of age. Data from an age of 8 to 36 months are represented by the mean ± SEM of two diploid groups and two triploid groups. Data at an age of 47 months are represented as the mean ± SEM of a diploid group and a triploid group. Asterisks indicate significant differences ($P < 0.05$). Black bars indicate the spawning periods.

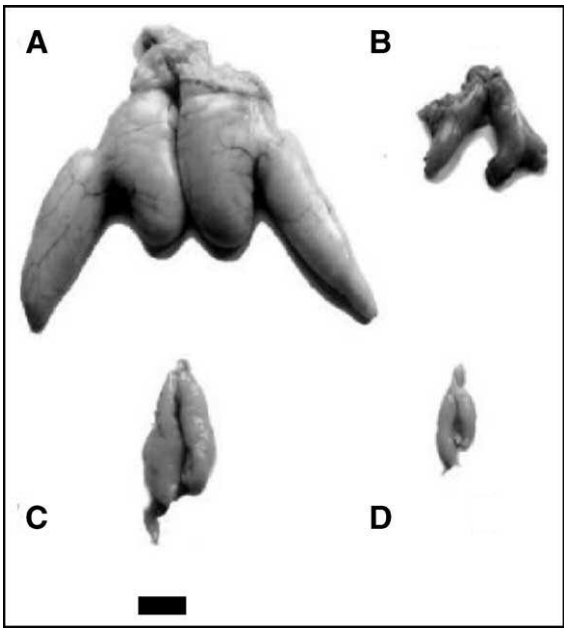

Fig. 5. Diploid turbot gonads: (A) females and (C) males, and triploid turbot gonads: (B) females and (D) males at 47 months of age. Bar=2.4 cm.

ed by the post-spawning mortality usually observed in regular diploids of this species.

During the juvenile phase and puberty, triploidy did not represent any clear advantage in terms of length and weight and thus triploids grew similarly to diploids, without any morphologic abnormalities being observed. This agrees with what has been described for other teleost species (Benfey et al., 1989; Felip et al., 2001b; Carrasco et al., 1999). In contrast, after the first spawning period (~ 24 months, ~ 1600 g) and until the end of the experiment, average triploid weight was slightly greater than diploid weight, with significant differences during the post-spawning periods due to the marked decrease in weight in the diploids – especially in females – caused by gonadal regression. These differences disappeared when the new sexual cycle started, due to the fast increase in the diploids gonadal weight. This is consistent with the existence of cycles of weight increase and decrease associated to the sexual cycle, which is quite marked in turbot – especially in females – due to the value of their gonadsomatic index (Suquet et al., 1994). Thus, diploid individuals rapidly gained weight during the months prior to spawning but lost nearly the same weight after spawning. In contrast, and due to the sterility of triploids – especially of females – this was hardly noticed, and thus triploids kept a nearly regular growth throughout the experiment, exhibiting a mean weight on average a 11.4% higher than that of diploids from 24 to 48 months of age.

The sex ratio of diploids (1 M:0.6 F) in this experiment was significantly different from that of triploids (1 M:3.3 F), and also to that previously described for diploid turbot (1 M:1 F) by Imsland et al. (1997). The higher number of females in the triploids also represented an additional advantage because, like in diploids (Imsland et al., 1997), triploid females reached a greater weight than triploid males. The increase in the number of females in triploids has previously been observed in other species such as sea bass (Felip et al., 2001b) and catfish (Goudie et al., 1995), probably reflecting changes in the relative amount of inherited paternal and maternal sex determining or related genes. Nevertheless, the higher proportion of females in triploid turbot should be confirmed in additional experiments. Sex proportions observed in triploids can also be due to a differential mortality (Benfey, 1999). The high percentage of sterile females in the triploids had a bearing on the outcome in terms of biomass after the third and during the fourth year of age. Triploids biomass at an age of 47 months was 10.3% or 14.3% higher than that of diploids – considering the total or eviscerated weight respectively – despite the fact that, at that time, immediately after spawning, female diploids weight was greater than that of triploid females. In addition, the higher predominance of females also reduced the size dispersion that it is usually observed in diploids. In the case of diploids, size dispersion between fish that mature and fish that do not mature, as well as between males and females, requires a continuous size selection.

The importance of sex control in species such as turbot where females grow more than males has prompted experiments aimed at controlling sex differentiation by either temperature manipulation, e.g., in *Paralichthys lethostigma* (Luckenbach et al., 2003), *Paralichthys olivaceus* (Yamamoto, 1999), or by hormonal treatment, e.g., in *Hippoglossus hippoglossus* L. (Hendry et al., 2003).

Temperature influence on the sex proportion in turbot has not been documented yet. Baroiller et al. (1998) considers that, although balanced sex ratios are generally observed in turbot, which suggests a monofactorial sex determination, the skewed male proportions observed in some cultures would indicate the influence of different factors, such as temperature. Nevertheless, according to unpublished data (A. Riaza, Stolt Sea Farm, personal communication) as well as to our own data (F. Piferrer and J. Fernández, unpublished observations), it seems that temperature has little or no influence on turbot sex ratios.

Gynogenesis induction in species that have homogametic females, such as *Paralichthys lethostigma*, can be used to create all-female stocks (Luckenbach et al., 2004). Experiments with this aim in turbot have resulted in 75% (Cal et al., 2002) or 100% females (Cal et al., submitted for publication). Since 100% females can only be obtained in species with female homogamety, and the occasional presence of males in some gynogenetic batches is common even in these species (Felip et al., 2001a), we have postulated that the turbot, like some other flatfish, has female homogamety (Cal et al., submitted for publication). For this reason, the use of gynogenetic neomales as sperm donors is an option that is currently being studied, as they can be mated with normal females to produce all-female stocks.

In summary, although the induction of triploidy does not result in a significant increase in size per se, by increasing the number of females, it does show interesting advantages, particularly in the rearing of turbots of more than 25 months of age. These include increased biomass as well as greater homogeneity in fish sizes because of the high percentage of females. Triploid turbot are also sterile, thus avoiding post-spawning mortalities, which also facilitates the fish marketing at any time of the year. Finally, sterility also means that triploids would not be able to breed with conspecifics, which constitutes an additional advantage, since this would avoid any adverse ecological effect of possible escapees. Thus, triploidy induction in turbot constitutes an interesting option, as it facilitates the production of fish of more than 2 kg, which are better valued and not affected by complications related to sexual maturity.

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Artículo 4

Effect of triploidy on turbot haematology

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Abstract

This study was carried out to compare key haematological features of diploid (2n) and triploid (3n) turbot as a first step towards the assessment of the ability of 3n turbot to withstand sub-optimal culture conditions. Morphometric indices of erythrocytes were determined on blood smears by light microscopy. Triploidy significantly ($P < 0.001$) increased all morphometric indices measured in the erythrocytes, including size, surface, and volume, except for the size of minor nuclear axis. The increase in cell size was larger for the major (31.0%) than for the minor (8.3%) axis, thus rendering erythrocytes of 3n turbot more ellipsoidal. The increase in erythrocyte volume (45.9%) was close to the theoretical expected 50% increase as a result of one extra chromosome set. Haematological indices were measured automatically by a haematological Coulter Counter. Triploid turbot had lower numbers of red blood cells (RBC: 1.84 cells pL^{-1} in 2n vs. 1.27 cells pL^{-1} in 3n; $P < 0.001$) but they were of a larger size (Mean corpuscular volume [MCV]: 145.51 fL in 2n vs. 181.78 fL in 3n; $P < 0.001$). However, the decrease in RBC was not compensated by the increase in MCV, and thus, triploidy decreased the haematocrit (Hct: 26.80% in 2n vs. 23.11% in 3n; $P < 0.001$) and total blood haemoglobin concentration (Hb: 73.74 g l^{-1} in 2n vs. 67.54 g l^{-1} in 3n; $P < 0.05$). In contrast, mean corpuscular hemoglobin (MCH: 40.27 pg in 2n vs. 53.28 pg in 3n; $P < 0.001$) was higher for 3n turbot as a result of their larger erythrocytes although MCH concentration (MCHC: 0.28 pg fL^{-1} in 2n vs. 0.29 pg fL^{-1} in 3n did not significantly differ. RBC, Hct and MCV were also determined manually using light microscopy. In general, discrepancies between the two methods were small (overall ~7%) but the Coulter Counter tended to overestimate RBC and Hct (and thus to underestimate MCV). Nevertheless, relative differences between ploidies were very similar, thus verifying triploidy-associated changes in hematological features. These changes, as determined in the present study, are essential when evaluating the feasibility of triploid turbot for intensive aquaculture systems in which unfavorable situations may occur.

Keywords: Turbot; Triploidy; Blood; Erythrocyte; Haematology; Haemoglobin; Oxygen transport

1. Introduction

The induction of triploidy has been achieved in a number of different freshwater and marine fish species (Thorgaard, 1983; Benfey, 1989; Ihssen et al., 1990; Felip et al., 2001a). The main benefit of triploidy is sterility condition. Sterility avoids metabolic costs of sexual maturation and as a result somatic growth continues in triploid fish, with maintenance of flesh quality during the period when diploids sexually mature. In addition, sterility avoids fish mortality related to spawning (Utter et al., 1983; Mair, 1993; Ihssen et al., 1990; Benfey, 1999). Because of these advantages, the induction and rearing of triploid fish is practiced in the aquaculture of several economically relevant species (Hulata, 2001). In addition, the use of sterile triploid fish has been suggested as a means of minimizing the danger that escapees pose to wild populations (Donaldson et al., 1993).

The turbot, *Scophthalmus maximus* L., is one of the most important species in European aquaculture. In the

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turbot, triploidy induction is an interesting option since sexual maturity is achieved before fish reach market size. The optimal conditions for the induction of triploidy in the turbot have been recently established (Piferrer et al., 2000, 2003). Furthermore, a 4-year study on the survival and growth of triploid turbot showed that (1) there were no apparent differences in survival between diploids and triploids; (2) triploidy per se did not increase growth of turbot. However, triploidy significantly increased the proportion of females from one half to two thirds of the population. Females are the fastest growing sex in turbot. By virtue of having more females, triploid turbot biomass was ~14% higher than the control diploids when fish were in the range 1.7–3.5 kg (Cal et al., 2005). This is the size range where the mean weight of triploid turbot is significantly higher than that of diploids. Thus, the induction of triploidy offers an interesting opportunity and an added value for increased production of medium- and large-sized turbot.

Morphologically, triploid fish are usually indistinguishable from their diploid counterparts. However, by virtue of having an extra chromosome set, triploidy causes an increase in cell size with a concomitant reduction in cell numbers in a variety of cell types, including erythrocytes (Benfey, 1999). The reason behind the reduction in cell numbers is still not well understood. Since erythrocytes are responsible of transporting oxygen from the external medium to the cell, an alteration of their number, size and, therefore, some inherent biochemical characteristics might compromise the ability of triploids to utilize oxygen. This may not be always apparent but can be of relevance in certain circumstances, e.g., under sub-optimal rearing conditions due to, for example, high water temperature or excessive fish rearing density. In turn, a lower capacity to utilize oxygen might reduce overall fish welfare and consequently appetite and level of immunity. Previous studies on the subject have reported that triploid fish might be more susceptible to the stress associated with routine management practices in fish farms as well as to diseases (Benfey and Sutterlin, 1984; Aliah et al., 1991). Preliminary work with the turbot has shown that triploids have a lower ability to react to acute hypoxia than their diploid homologues (Cal et al., 2001).

The present study was conducted therefore to compare some key haematological indices in diploid and triploid turbot. These data would be useful when evaluating the feasibility of intensive production of triploid turbot by the aquaculture industry.

2. Materials and methods

2.1. Fish

The turbot used were the same as those in which the induction of triploidy in this species was reported (Piferrer et al., 2000, 2003). Briefly, diploids and triploids belonged to the same initial batch of eggs, thus avoiding the possibility of strain- or family-associated differences in haematological indices. Fish were born at the facilities of the Instituto Español de Oceanografía in Vigo (NW coast of Spain) and were also used for an in-depth 4-year study of the survival, growth performance, and reproduction of triploid vs. diploid turbot (Cal et al., 2005). The ploidy level was verified by NOR analysis and erythrocyte size measurements as described in Piferrer et al. (2000, 2003).

Fish were cultured in four 3800-L tanks containing 100 fish each, two of them with diploids and two with triploids, with an open circuit of seawater, under natural conditions of temperature and photoperiod, and fed daily with dry industrial pellets containing 52.0% crude protein, 20.0% crude fat, 12.0% ash, 0.2% cellulose, and the following vitamins (per kilogram of diet): vitamin A, 10,000 I.U.; vitamin D_3, 1500 I.U.; vitamin E (α-tocopherol, 150 ng, plus antioxidant Etoxiquine) and minerals (Trouw, Burgos, Spain), using automatic feeders. Fish stocking density at the time of sampling was 21.5 kg m^{-3} for diploids and 20.3 kg m^{-3} for triploids. Water temperature when blood was obtained was 13.5 °C, and dissolved oxygen 7.4 ± 0.1 mg $O_2 L^{-1}$.

2.2. Blood samples

For the present study, 35 diploid and 24 triploid turbot of 18 months of age were used. Mass and length (mean ± S.D.) were 1120.0 ± 39.04 g and 37.0 ± 0.41 cm (TL) in diploids

Table 1
Erythrocyte dimensions in diploid and triploid turbot as assessed on blood smears by light microscopy

Variable (units)	Diploids (*D*) (*n* = 100)	Triploids (*T*) (*n* = 100)	Ratio (*T*/*D*)	Significance level[a]
Cell minor axis (μm)	8.45 ± 0.11	9.15 ± 0.09	1.08	$P < 0.001$
Cell major axis (μm)	11.80 ± 0.10	15.46 ± 0.09	1.31	$P < 0.001$
Cell surface (μm^2)	78.82 ± 1.58	111.09 ± 1.27	1.41	$P < 0.001$
Cell volume (fL)	444.10 ± 14.09	648.10 ± 13.45	1.46	$P < 0.001$
Nuclear minor axis (μm)	3.07 ± 0.08	3.20 ± 0.06	1.03	$P > 0.05$
Nuclear major axis (μm)	4.52 ± 0.08	6.34 ± 0.04	1.40	$P < 0.001$
Nuclear surface (μm^2)	11.26 ± 0.49	15.85 ± 0.30	1.41	$P < 0.001$
Nuclear volume (fL)	25.44 ± 1.98	35.03 ± 1.30	1.38	$P < 0.001$

Data as mean ± S.E.M.
[a] By Mann–Whitney test.

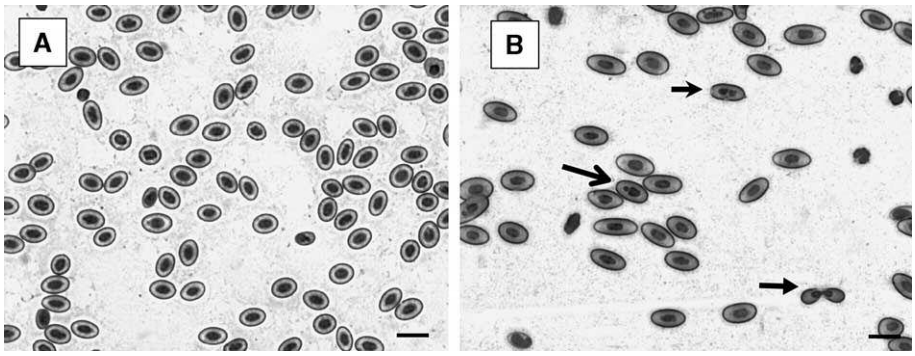

Fig. 1. Diploid (A) and triploid (B) turbot erythrocytes. Note the larger size and the occurrence of abnormalities of the triploid cells, including cells with bisected nuclei (stealth arrow), cell with micronucleus (open arrow), and pinched cell (arrow). Bar equals 12 μm.

vs. 1055.2±0.348 g and 36.9 ± 0.35 cm (TL) in triploids, without the existence of significant differences (Student's *t*-test; $P > 0.05$). Fulton's body indices (body mass in g×100. TL^{-3}) was 2.19 ± 0.031 in diploids and 2.08 ± 0.029 in triploids ($P > 0.05$). Fish were deprived of food during the 24 h previous to sampling and then blood samples were obtained by venipuncture in the branchial arch between 10:00 and 12:00 h during December 2000, using a heparinized 1-mL syringe. During the extraction, anesthetic products were not used, and to minimize stress, fish were deprived of visual stimuli by covering their head with a damp cloth, as normally done with this species.

2.3. Erythrocyte measurements

The outlines of 100 erythrocytes and their nuclei from 10 fish in each ploidy were determined from dry blood smears with the aid of a light microscope Axioskop 2 (Carl Zeiss Jena GmbH) (Piferrer et al., 2000). Dry blood smears were prepared from each fish by the conventional method, fixed in methyl alcohol and stained with eosin and tiacine. Erythrocyte and nuclear surface area were calculated as $S=\pi \cdot a \cdot b$, where *a* and *b* are the major and the minor semi-axis of the cell and of the nucleus, respectively. Erythrocyte and nuclear volume was calculated as $V=4/3\pi \cdot a \cdot b^2$, where *a* and *b* are the major and the minor semi-axis of the cell and of the nucleus, respectively. The cell and nuclear major and minor axis, surface and volume for each ploidy were compared.

2.4. Automatic measurement of haematological indices

Total red blood cell count (RBC) and mean corpuscular volume (MCV) were determined by means of a haematological Coulter (Coulter® Channelyzer model STKS, Coulter Corporation, Miami, FL, USA). Fresh whole-blood 250-μL samples were diluted 1:200 with Coulter Isoton III diluent (Beckman Coulter, Inc. Fullerton, CA). Haemoglobin concentration (Hb) was obtained using the same analyzer by the cyanmethemoglobin spectrophotometry method using a wavelength of 525 nm and specifications for international haemiglobincyanide reference preparation (International Committee for Standardization in Haematology, 1978). The RBC, MCV and Hb values were determined within 12 h following blood sampling.

From the previous parameters, haematocrit (Hct), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were obtained using the following formulas (Benfey and Sutterlin, 1984):

$$\mathrm{Hct} = \mathrm{MCV}\ (\mathrm{fL}) \times \mathrm{RBC}\ \left(\mathrm{cell\ pL^{-1}}\right)$$

$$\mathrm{MCH} = \mathrm{Hb}\ \left(\mathrm{g\ l^{-1}}\right)/\mathrm{RBC}\ \left(\mathrm{cell\ pL^{-1}}\right)$$

$$\mathrm{MCHC} = \mathrm{MCH}\ (\mathrm{pg})/\mathrm{MCV}\ (\mathrm{fL})$$

2.5. Manual measurement of haematological indices

To verify the results obtained with the haematological Coulter, RBC, Hct and MCV were also determined manually (RBC_m, Hct_m and MCV_m) on replicate blood samples from the same fish within 12 h following blood sampling. RBC_m was determined with a Neubauer haemocytometer after the blood was diluted 200 times with the Natt and Herrick's solution (Natt and Herrick, 1952). The total number of red blood cells was counted in the five sub-squares of the central large square of the chamber (Nelson and Morris, 1993). Hct_m was measured using 2–3 μL of refrigerated blood in heparinized microhaematocrit capillary tubes after centrifugation (2500 rpm, 5 min, 4 °C). MCV_m was calculated dividing Hct_m by RBC_m.

2.6. Data analysis

Major descriptive statistics were obtained for all the studied variables. Distributions were examined for departures from normality by the Kolmogorov–Smirnov test and the homogeneity of variances was verified by the Levene's test. Values for diploid and triploid fish were compared by using the *t*-test or by the Mann–Whitney test when the data did not have a normal frequency distribution (Sokal and

Table 2
Haematological indices in diploid and triploid turbot as assessed by automatic counting by an haematological Coulter Counter

Variable (units)	Diploids (*D*) (*n* = 100)	Triploids (*T*) (*n* = 100)	Ratio (*T*/*D*)	Significance level
RBC (cells pL^{-1})	1.84±0.043	1.27±0.024	1.45	$P<0.001$[a]
Hct (%)	26.80±0.624	23.11±0.594	1.16	$P<0.001$[b]
MCV (fl)	145.51±1.424	181.78±2.024	0.80	$P<0.001$[b]
Hb (g L^{-1})	73.74±1.921	67.54±1.935	1.09	$P<0.05$[b]
MCH (pg)	40.27±1.028	53.28±1.293	0.76	$P<0.001$[b]
MCHC (pg fL^{-1})	0.28±0.007	0.29±0.006	0.97	$P>0.05$[b]

Data as mean ± S.E.M.
Abbreviations: RBC, red blood cell number; Hct, haematocrit; MCV, mean corpuscular volume; Hb, haemoglobin; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration.
[a] By Mann-Whitney test.
[b] By *t*-test.

Rholf, 1981). All analyses were carried out using the statistical package SSPS version 10.1 (Imation Enterprises Corp. Chicago, IL, USA). Differences were accepted as significant when $P < 0.05$.

To check whether measurements of RBC, Hct and MCV carried out either automatically with the Coulter Counter or manually gave similar results and for possible bias, a scatter diagram of the differences between the two methods against the average RBC, Hct and MCV were made (Bland and Altman, 1986).

3. Results

3.1. Erythrocyte measurements

Triploidy significantly ($P < 0.001$) increased all variables measured in the erythrocytes, including size, surface and volume, except for the size of the minor nuclear axis ($P > 0.05$) (Table 1). The increase in cell size was larger for the major (31.0%) than for the minor (8.3%) axis, thus rendering erythrocytes of triploids more ellipsoidal. The increase in nuclear size was also larger for the major (40.3%) than for the minor (4.2%) axis. The increase in cell volume (45.9%) was similar as the increase in nuclear volume (37.7%).

The total incidence of abnormalities in erythrocytes was affected by ploidy. Nuclear changes (segmented nuclei, pinched cells and occurrence of micronuclei) were higher in triploids (9.1%) than in diploids (0.7%) ($P < 0.05$). Specifically, the incidence of erythrocytes with segmented nuclei was higher in triploids (7.7% in 3n vs. 0.7% in 2n) ($P < 0.05$), as was the incidence of pinched cells (1.2% in 3n vs. 0.0% in 2n) ($P < 0.05$) or micronuclei (0.2% in 3n vs. 0.05% in 2n) ($P < 0.05$) (Fig. 1).

3.2. Haematological indices

The haematological indices obtained with the Coulter (Coulter® Channelyzer STKS) are presented in Table 2. Haematological analysis with the Coulter showed a mean decrease of 31.5% for RBC and a mean increase of 24.9% for MCV in triploids ($P < 0.001$). These two changes combined resulted in that the Hct of triploids was on average 13.8% lower ($P < 0.001$). Furthermore, the Hb of triploids was 8.5% lower ($P < 0.05$), whereas their MCH was 32.2% higher ($P < 0.001$). The MCHC was 3.6% higher in triploids but the difference was not significant ($P > 0.05$).

Similar results were found when comparing RBC, Hct and MCV values of diploids and triploids obtained from manual measurements (RBC_m, Hct_m and MCV_m; Table 3). When compared to diploids, in triploids the RBC_m decreased 25.1% ($P < 0.001$), Hct decreased 6.3% ($P < 0.05$), and MCV increased 24.1% ($P < 0.001$).

It was found that the value of RBC and Hct were overestimated when using the automatic procedure as compared to manual determination, and that for RBC and Hct the magnitude of the overestimation was higher for diploid than for triploid fish.

No relationship between the difference and the average values for RBC and Hct was apparent from the scatter plots (Fig. 2A–D), although a certain downward trend was observed in the plot of difference and mean RBC (Fig. 2A). In contrast, the discrepancy between automatic and

Table 3
Haematological indices in diploid and triploid turbot as assessed by manual counting using a Neubauer haemocytometer and microhaematocrit capillary tubes

Variable (units)	Diploids (*D*) (*n* = 100)	Triploids (*T*) (*n* = 100)	Ratio (*T*/*D*)	Significance level[a]
RBC (pL^{-1})	1.59 ± 0.047	1.19 ± 0.038	1.34	$P<0.001$
Hct (%)	24.89 ± 0.505	23.31 ± 0.524	1.07	$P<0.05$
MCV (fL)	160.09 ± 4.099	198.68 ± 6.410	0.81	$P<0.001$

Abbreviations as in Table 2.
[a] By *t*-test.

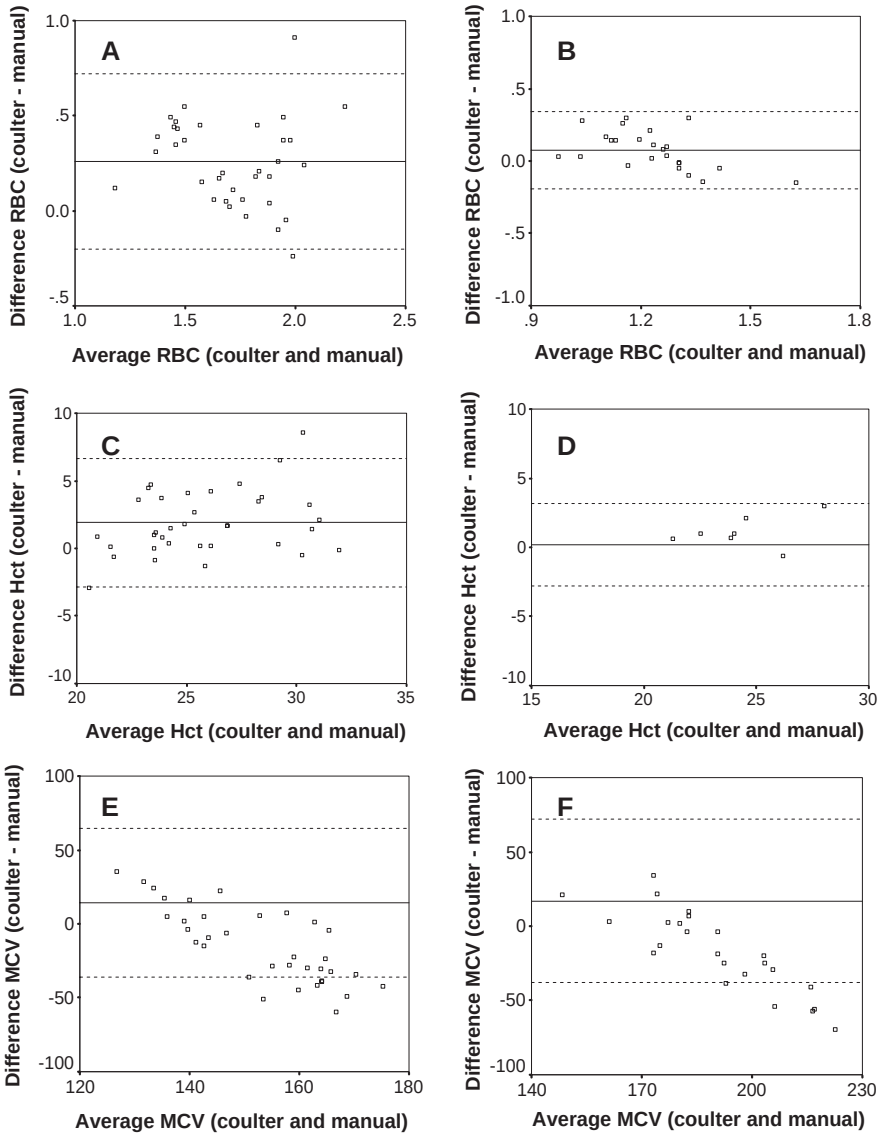

Fig. 2. Scatter plots of differences against mean for RBC (A and B), Hct (C and D) and MCV (E and F) as determined automatically or manually in diploid (A, C, E) and triploid (B, D, F) turbot. The solid lines indicate the mean and the dashed lines ± 2 S.D.

manual estimates of MCV was negatively correlated to the mean MCV (Fig. 2E–F). For low values of MCV, the manual procedure gave lower values than the Coulter, while for high MCV the opposite was true.

4. Discussion

In diploid turbot, erythrocytes constitute approximately 94% of the total blood cells (Quentel and Obach, 1992). As in other species of fish studied so far, in the turbot triploidy increased erythrocyte size. This increase did not affect equally all measurements since it was larger for the major (longitudinal) than for the minor (transversal) axis. This is consistent with the fact that the major axis of fish erythrocytes appears to be more affected by triploidy (Wattendorf, 1986; Kim et al., 1994; Benfey, 1999), hence altering the shape of the cell, which becomes more ellipsoidal. Likewise, the relationship between erythrocyte and nuclear size was significantly correlated in diploid and triploid turbot, in agreement with what occurs in most teleost species studied so far (Hardie and Hebert, 2003).

The influence of triploidy on the segmentation of erythrocyte nuclei and on the occurrence micronuclei was also observed in the present study. The segmentation is a regular division of nuclei of mature erythrocytes (Yokote, 1982). The presence of segmentation in the nuclei of erythrocytes has been discussed by Benfey (1999) as a consequence of triploidy and observed in other species, e.g., in brook trout, *Salvelinus fontinalis* (Wlasow et al., 2004). Erythrocytes with divided nuclei were also observed in folic acid-deficient coho salmon, *Oncorhynchus kisutch* (Smith, 1968). Likewise, the higher occurrence of micronuclei in triploids than in diploids was reported by Strunjak-Perovic

et al. (2003) in the brook trout and by Anitha et al. (2000) in adult goldfish (*Carassius auratus*) subjected a heat shock. These alterations can occur as a consequence of both structural and numerical chromosomal aberrations (Miller et al., 1998), as in the case of triploids, but also as a consequence of heat thermal shock. Since erythrocytes of triploids are in fact polyploid cells, they potentially contain more molecular targets for genetic damage during cell division in comparison with those of diploids (Strunjak-Perovic et al., 2003).

The observed values of MCV, Hb and nuclear volume in this study fall within the range of values previously reported for fish: MCV, 75.0–317.9 fL; Hb, 35.0–130.9 g L^{-1}; nuclear volume, 7.3–46.2 fL (Lay and Baldwin, 1999). The increase in erythrocyte volume (45.9%) was close to the theoretical increase that one would expect as a result of carrying one extra chromosome set and thus of having 50% more DNA (Benfey, 1999).

In some aspects, triploidy affected turbot haematology differently than it does to some other fish species. Thus, although in triploid turbot the increase in cell size (MCV) was accompanied by a reduction in cell number (RBC), still the Hct of triploids was lower than that of diploids. This contrasts with the situation in other species, i.e., the Atlantic salmon, *Salmo salar,* and the sea bass, *Dicentrarchus labrax*, among others, where the increase in erythrocyte size was compensated by a proportional reduction in its numbers, thus resulting in a similar Hct (Benfey and Sutterlin, 1984; Sezaki et al., 1991; Biron and Benfey, 1994; Yamamoto and Lida, 1994; Sadler et al., 2000; Felip et al., 2001b; Rehulka et al., 2004). The constitutively lower Hct of turbot when compared to those of most fishes may be explained by the fact that this flatfish is of low swimming activity, and that spends most of its time lying on the sand in the seabed.

An increased MCV is associated with a decrease in the surface area-to-volume ratio of triploid red blood cells which, along with a reduced Hct, might have a marked effect on the physiology of triploid turbot, since the total area available for oxygen assimilation will be reduced (Graham et al., 1985). Other processes also limited by the surface area such as nutrient or ionic exchange might also be affected. The changes observed in the shape of the cell and its nucleus are also likely to affect oxygen assimilation in triploid turbot due to an increase in diffusion distances within the erythrocyte (Benfey, 1999).

In triploid turbot, Hb content of the erythrocytes was higher than in diploids as a result of their larger volume. However, total blood concentration of Hb was lower in triploids. Haemoglobin content of the erythrocyte is crucial for oxygen distribution to the different tissues and its blood concentration is considered as an indicator of the ability of the organism to incorporate the oxygen needed for its metabolic processes (Benfey, 1999). This reduction in the ability of triploid red blood cells to oxygenate could theoretically result in a reduced aerobic capacity, but this has not been definitively demonstrated in any species (Benfey and Sutterlin, 1984; Sezaki et al., 1991; Yamamoto and Lida, 1994). Further, Sadler et al. (2000) found that triploid Atlantic salmon despite having fewer, larger erythrocytes, had a very similar oxygen carrying capacity as diploids, indicating that farm mortalities of triploids in response to stress is not due to failure in respiratory homeostasis.

It might be speculated that the reduced oxygenation efficiency at the blood cell level is not of magnitude enough to alter whole animal oxygen requirements (Graham et al., 1985). Graham et al. (1985) and Stillwell and Benfey (1996) suggested that it is possible to achieve an optimum development of triploid fishes in oxygen-rich environments but this might be compromised in oxygen-deficient environments due to the lower carrying capacity of their erythrocytes. Consistent with this view, this would explain the lower tolerance to acute hypoxia of triploid turbot with respect to diploids (Cal et al., 2001).

Compared to manual measurement and counting, the automated method consistently overestimated RBC and Hct. A possible explanation for this may be the erroneous count as red blood cells of a certain proportion of white blood cells by the Coulter. This would explain the downward trend (Fig. 1A), since the higher the RBC value, the lower the discrepancy. Since RBC results are used by the Coulter to estimate Hct, this could also explain the lower value of Hct of diploids (Fig. 1C) when measured manually. It should be emphasized that differences between the two methods are unlikely to have any bearing on the results of our comparisons between diploids and triploids, because, first, such comparisons were based on both methods, and second, because the detected bias was in the same direction regardless of ploidy level. However, our results suggest that automatic haematological counters such as the Coulter should only be used after careful calibration against accepted tests or gold standards. The overestimation of MCV by automatic methods is higher for higher MCV values. A possible explanation for this could be an incorrect adaptation of the Coulter's discriminators in case of high MCV that could lead to leukocytes being erroneously measured as erythrocytes.

Hematological studies have been performed in many fish species but few of them are of flatfishes. The results of this study add heamatological information for a new species of flatfish, which contributes to alleviate this situation. Our results showed triploidy-associated changes in haematological parameters which might affect turbot physiology, specifically the capacity of this fish to use oxygen in low concentration conditions and thus to react to acute hypoxia. Current farming practices such as in farm continuous monitoring of water quality and the deliberate use of oxygen saturation conditions in the rearing tanks certainly would minimize the impact of triploidy-induced changes in oxygen use capabilities. Nevertheless, these changes, as they have been determined in the present study, should be taken into account when assessing the feasibility of triploid turbot for intensive aquaculture systems where unfavorable rearing situations may occur.

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CAPÍTULO III
LA GINOGÉNESIS EN EL RODABALLO

LA GINOGÉNESIS EN EL RODABALLO

El estudio de la ginogénesis en el rodaballo se realizó con el fin de estudiar la posibilidad de producir poblaciones todo hembras, que en esta especie representaría una gran ventaja debido a su mayor crecimiento y su maduración más tardía.

Este estudio se desarrollo en tres fases. En la primera se desarrollo el método de inducción de la ginogénesis (Artículo 5), en la segunda se determinó la herencia exclusivamente materna en la descendencia (Artículo 6), y en la tercera se determinó el crecimiento, el desarrollo gonadal y la proporción de sexos en rodaballos ginogenéticos diploides (Artículo 7).

Resumen: La ginogénesis en el rodaballo. (*Scophthalmus maximus*). Inducción, verificación, efectos sobre el crecimiento y la reproducción.

Artículo 5. Piferrer, F., **Cal, R.M.,** Gómez, C., Álvarez-Blázquez, B., Castro, J. and Martínez, P. 2004. Induction of gynogenesis in the turbot (*Scophthalmus maximus*): Effects of UV-irradiation on sperm motility, the Hertwig effect and viability during the first 6 months of age. **Aquaculture**, 238: 403-419.

Artículo 6. Castro, J., Bouza, C., Sánchez, L., **Cal, R.M**., Piferrer, F. and Martínez, P. 2003. Gynogenesis assessment by using microsatellite genetic markers in turbot (*Scophthalmus maximus)*. **Marine Biotechnology***,* 5: 584-592.

Artículo 7. **Cal, R.M**., Vidal, S., Martínez, P., Álvarez-Blázquez, B., Gómez, C. and Piferrer, F. 2005. Growth and gonadal development of gynogenetic diploid turbot (*Scophthalmus maximus*). **Journal of Marine Biology** (en prensa).

LA GINOGÉNESIS EN EL RODABALLO (*Scophthalmus maximus*): INDUCCIÓN, VERIFICACIÓN Y EFECTOS SOBRE EL CRECIMIENTO Y LA REPRODUCCIÓN

El rodaballo (*Scophthalmus maximus*) es una especie de importancia creciente en la acuacultura europea. Su crecimiento está afectado por el sexo y por la maduración. Los machos crecen menos que las hembras a partir de los 8 meses desde la eclosión (Imsland, 1997) y este crecimiento diferencial se mantiene durante el resto del ciclo de producción incluyendo la maduración sexual. Así mientras las hembras maduras pueden alcanzar 1,8 Kg en 20 meses, los machos alcanzan alrededor de 1 Kg. Por este motivo se ha sugerido que, como ya se hace en otras especies, se debería desarrollar un método para producir poblaciones todo hembras en el rodaballo (Imsland et al., 1997), pero hasta la fecha no hay datos disponibles sobre métodos hormonales, directos o indirectos, para producir poblaciones todo hembras en esta especie.

En peces se pueden producir poblaciones de todo hembras por tratamiento hormonal con estrógenos, para feminizar a peces sexualmente indiferenciados (Piferrer, 2001). Sin embargo, aunque los esteroides están permitidos para el control del sexo durante los primeros estados de desarrollo en peces en la legislación de muchos países, esta práctica produce rechazo en los consumidores. La ginogénesis, junto con la producción de neomachos (hembras genéticas/machos fisiológicos) es un método indirecto que puede ser aplicado para obtener progenie todo hembras, cuando las hembras son el sexo homogamético (Piferrer, 2001).

La ginogénesis es una técnica de manipulación cromosómica que consiste en la generación de progenie cuyos cromosomas son herencia exclusivamente materna (Chourrout, 1982; Thorgaard, 1983). La inducción de la ginogénesis incluye la desactivación del ADN del esperma, mientras se mantiene su capacidad para desencadenar el desarrollo embrionario.

El resultado son embriones haploides y no viables tras la eclosión, a menos que se haya restaurado la diploidía, reteniendo el segundo corpúsculo polar o inhibiendo la primera división mitótica (Thorgaard, 1983). La inducción de la ginogénesis resulta en un bajo porcentaje de peces viables a causa de la manipulación aplicada pero el resultado suele ser poblaciones todo hembras cuando estas son el sexo homogamético (Devlin and Nagahama, 2002). En la práctica, la ginogénesis no asegura siempre el 100% de hembras en la descendencia, aunque si un alto porcentaje (Felip et al., 2001a).

A causa de la baja viabilidad de los ginogenéticos, una práctica alternativa es cambiar de sexo a las hembras ginogenéticas para obtener neomachos (individuos genéticamente hembras y fisiológicamente machos) productores de esperma, para fertilizar huevos de hembras naturales y así producir poblaciones todo hembras (Piferrer et al., 1994; Donaldson 1996; Felip et al., 2001a).

En este estudio el objetivo fue establecer un protocolo para producir rodaballos ginogenéticos viables combinando la activación del huevo con esperma irradiado con UV, con la aplicación de un choque frío a los huevos recién activados. Este estudio se desarrolló: 1) investigando el efecto de la luz UV sobre el esperma del rodaballo, con respecto a su capacidad para fertilizar (activar) los huevos y desencadenar el desarrollo embriónico, 2) determinando las condiciones óptimas para inducir ginogénesis en el rodaballo, combinando la activación de los huevos con esperma irradiado con UV, con la aplicación de un choque frío a los huevos recién activados, 3) estableciendo un método para evaluar la herencia exclusivamente materna en la descendencia de ginogenéticos, 4) estudiando la viabilidad y el desarrollo de la progenie obtenida hasta adultos.

Los gametos para estos experimentos se obtuvieron por masaje abdominal de un lote de reproductores mantenido en un fotoperiodo de 16 h de luz y 8 h de oscuridad, a temperatura entre 13-14ºC en las instalaciones del Instituto Español de Oceanografía de Vigo.

La inducción de la ginogénesis se realizó inactivando el DNA del esperma antes de la fertilización, eliminando así la participación del genoma paterno en la descendencia. Se realizaron cuatro experimentos:

En el experimento 1, se examinó la calidad del esperma según el macho donante y el efecto de la dilución sobre la capacidad de fertilización, así cómo el efecto de la radiación UV sobre la movilidad del esperma. Los resultados mostraron que el porcentaje de fertilización depende más de la calidad del esperma que de la dilución, y que normalmente una dilución 1:10 es adecuada para inactivar el ADN del esperma.

La aplicación de la dosis adecuada de radiación para desactivar el ADN del esperma, mientras se mantiene su capacidad para desencadenar el desarrollo del embrión, es el punto crítico de la inducción a la ginogénesis (Felip et al., 1999). El rodaballo es un bajo productor de esperma y sus larvas son pequeñas y frágiles, lo que representa un inconveniente añadido para la inducción de la ginogénesis. Dosis crecientes de radiación disminuyeron la movilidad del esperma tanto en el número de espermatozoides móviles como en la duración de la movilidad. La dosis a la cual tanto la movilidad del esperma como la duración de la movilidad se redujo al 50% del valor original (ID_{50}) fue ~28 000 erg.mm^{-2}.

En el experimento 2, se determinó el efecto Hertwig, (dosis de radiación necesaria para la total desactivación del esperma sin comprometer su capacidad para desencadenar el desarrollo embrionario). El típico efecto Hertwig se produjo con una dosis de 30 000 erg.mm^{-2}. Los embriones resultantes mostraron el típico "síndrome haploide" y murieron poco después de la eclosión. La aplicación de un choque frío (–1 a 0°C durante 25 minutos, iniciada 6.5 minutos después de la fertilización) a los huevos fertilizados con esperma diluido (1:10) e irradiado con UV (30 000 erg mm^{-2}), restableció la diploidía y tuvo como consecuencia la producción de rodaballos ginogenéticos diploides (2n = 44 cromosomas).

En los experimentos 3 y 4, se utilizaron estas condiciones para inducir ginogénesis en un experimento a escala piloto y en otro a gran escala. La tasa de inducción de ginogénesis fue del 100%, conforme a lo verificado mediante análisis con marcadores de DNA microsatélite, pero la supervivencia de los ginogenéticos hasta los 22 días de vida fue significativamente menor ($P<0,05$) que la de los controles no tratados. A los 6 meses de edad la supervivencia fue similar entre los controles y los ginogenéticos, y no se registró diferencia estadísticamente significativa en el crecimiento en talla y peso.

La ploidía fue inicialmente determinada en larvas de un día de vida y en embriones en el caso de ginogenéticos haploides, por análisis de cariotipo y por análisis de NOR. En los cariotipos, todos las extensiones mostraron el nivel de ploidía esperado, tanto en los ginogenéticos haploides (n) como en los ginogenéticos diploides (2n) y como en los grupos control. Las metafases de embriones de huevos fertilizados con esperma irradiado fueron haploides (22 cromosomas). Los potenciales ginogenéticos diploides mostraron el cariotipo estándar del rodaballo (44 cromosomas), igual que las larvas del grupo control obtenidas de huevos fertilizados con esperma normal. Los análisis de cariotipo en embriones y larvas demostraron alta precisión en la estimación del nivel de ploidía, pero el rendimiento fue limitado dado el bajo número de buenas preparaciones obtenidas.

En los análisis de NOR, las distribuciones de frecuencias del número de nucleolos por célula en diploides y triploides estuvieron solapadas en uno de los dos cruzamientos efectuados, rindiendo aproximadamente un 3% de error en la determinación del nivel de ploidía. La utilización de esta técnica es válida para estimar el éxito de una particular manipulación de la ploidía, aunque los resultados deben ser considerados con precaución. Pero en ginogenéticos no es aplicable, ya que aunque el porcentaje de clasificación errónea es pequeño, el análisis de NOR no garantiza la identificación de paternidades lo cual es esencial en el establecimiento de un lote de reproductores ginogenéticos en rodaballo.

La herencia exclusivamente materna de los ginogenéticos, fue determinada sin ambigüedades utilizando marcadores microsatélite. El DNA fue extraído de las muestras de aleta obtenidas de ambos padres en cada cruzamiento, y de todo el individuo en la progenie (embriones y larvas) usando resina Chelex 100 según Walsh et al. (1991). La reacción en cadena de la polimerasa (PCR) fue realizada con cebadores específicos diseñados para amplificar 11 microsatélites de rodaballo previamente caracterizados (Coughlan et al., 1996; Estoup et al., 1998; Bouza et al., 2002).

Según los genotipos determinados previamente por microsatélite en ambos padres, en cada cruzamiento dos loci diagnóstico fueron seleccionados entre 11 analizados y amplificados en 15-20 potenciales ginogenéticos en cada familia. El uso de un conjunto de 11 loci microsatélites altamente variables resultó en un método potente para confirmar la existencia de la herencia exclusivamente materna en los descendientes ginogenéticos en el rodaballo, con probabilidad de detección de la posible contribución genética paterna por encima del 99.99%. Con este nivel de exclusión, cualquier contribución paterna hubiera podido ser detectada en los ginogenéticos una vez conocido el genotipo materno.

El estudio del desarrollo de los rodaballos ginogenéticos se determinó en un grupo de 33 rodaballos ginogenéticos diploides y 33 rodaballos diploides (control) procedentes todos de la misma puesta a partir de los de 6 meses de edad.

Los peces se cultivaron de forma similar pero por separado. Al inicio del experimento todos los peces fueron individualizados marcándolos con microchips. Periódicamente todos los peces se midieron y se pesaron para determinar la tasa de crecimiento y el índice de condición. A los 24 y a los 36 meses de edad una muestra de cada grupo fue sacrificada y se determinó su desarrollo gonadal.

Entre los 9 y los 36 meses de edad, no hubo diferencia significativa de la supervivencia entre el control diploide y los ginogenéticos diploides.

La longitud y el peso medio fueron mayores en los controles que en los ginogenéticos, no obstante, esta diferencia sólo fue significativa en longitud en los periodos desde 15 a 30 meses de edad en hembras, y desde 12 a 24 meses para machos y en peso desde 21 a 24 meses de edad en hembras y desde los 15 a los 21 meses de edad en machos.

A los 24 meses de edad, durante la primera maduración sexual, los machos ginogenéticos y control mostraron las gónadas bien desarrolladas, con activa espermatogénesis y espermatozoides. Sin embargo, mientras las hembras del control mostraron oocitos maduros, las hembras ginogenéticas solo mostraron oocitos en estado perinuclear. A los 36 meses, se observo un desarrollo gonadal completo en ambos sexos de ambos grupos. Así, mientras en los machos ginogenéticos el desarrollo gonadal sucedió normalmente, en las hembras ginogenéticas el desarrollo gonadal estuvo retrasado durante la primera maduración sexual, pero fue normal durante la segunda.

La fertilidad de los gametos de los ginogenéticos fue demostrada realizando una fertilización de un grupo de huevos de hembra ginogenética con esperma de un macho ginogenético, obteniéndose embriones que se desarrollaron normalmente hasta eclosionar.

La proporción de sexos en un primer experimento fue 1M: 1H en los controles, y de 1M:3H en los ginogenéticos. En un segundo experimento realizado con peces de un lote diferente, la proporción de sexos fue 1M:1H en los controles y 0M:1H (100% hembras) en los ginogenéticos.

Pero una descendencia 100% hembras ginogenéticas sólo puede ser obtenida en especies en las cuales las hembras son el sexo homogamético. En conjunto, estos resultados sugieren que el principal componente de la determinación genética del sexo en el rodaballo se ajusta bien al de hembras homogaméticas, como en otros peces planos, y que se pueden obtener lotes de todo o casi todo hembras a través de la inducción de la ginogénesis.

La utilización de los ginogenéticos masculinizados mediante tratamiento hormonal (Neomachos), portadores de herencia materna, como donadores de esperma, permitiría al fertilizar huevos de hembras normales la producción de una descendencia del 100% de hembras.

Otra aplicación de la ginogénesis es el cruzamiento de líneas altamente consanguíneas para explotar el componente dominante de la varianza genética (Purdon, 1983; Tave, 1993), aunque la consanguinidad disminuye la viabilidad, pudiendo ir también en detrimento de otras características también importantes.

La ginogénesis tiene aplicaciones en investigación básica. Por ejemplo, suministra información importante para conocer el mecanismo de determinación sexual de una especie dependiendo de la proporción de sexos de los ginogenéticos en la descendencia, y además los ginogenéticos haploides son una fuente de DNA importante en la construcción de mapas genéticos.

Artículo 5

Induction of gynogenesis in the turbot (*Scophthalmus maximus*): Effects of UV irradiation on sperm motility, the Hertwig effect and viability during the first 6 months of age

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Induction of gynogenesis in the turbot (*Scophthalmus maximus*): Effects of UV irradiation on sperm motility, the Hertwig effect and viability during the first 6 months of age

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Abstract

Fish in which gynogenesis has been induced have all their chromosomes inherited from the mother and, if females are the homogametic sex, they usually are all females. Because turbot females grow faster than males, the production of all-female populations is highly desirable. The sperm of turbot is of poor quality and its larvae are small and fragile. These circumstances represent a challenge for the induction of gynogenesis in the turbot. As a first step towards this goal, effective conditions for the induction of gynogenesis through UV irradiation of sperm followed by a cold shock were established. When diluted 1:10 with Ringer-200 saline solution and placed in a thin layer (~ 0.3 mm), a dose-dependent effect of UV light on sperm motility was found. The dose at which both the amount of motile sperm and the duration of sperm motility was reduced to 50% of the original value (ID_{50}) was ~ 28,000 erg mm^{-2}. A typical Hertwig effect was elicited with a dose of 30,000 erg mm^{-2}. The resulting embryos exhibited the typical "haploid syndrome" and died shortly after hatching. Application of a cold shock (− 1 to 0 °C for 25 min starting at 6.5 min after fertilization) to activated eggs with UV-irradiated (30,000 erg mm^{-2}) and diluted (1:10) sperms restored diploidy and resulted in the production of gynogenetic diploids ($2n = 44$ chromosomes). These conditions were used in a pilot-scale experiment and found effective in inducing gynogenesis

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in ~47,000 eggs. The rate of gynogenesis induction was 100% as verified by an analysis with microsatellite DNA markers. Survival of the gynogenetics was approximately 10% of diploids at 6 months of age, although growth was similar during this period. If this species turns out to have female homogamety, as is the case in most pleuronectiformes examined so far, the method presented here is the first necessary step for the production of all-female populations of this economically important species.

Keywords: Gynogenesis; UV irradiation; Cold shock; Sperm motility; Hertwig effect; Turbot; *Scophthalmus maximus*; Sex control

1. Introduction

Turbot (*Scophthalmus maximus*) is a species of growing importance for European aquaculture. Its production has steadily increased from 2966 mT in 1995 (FAO, 1997) to 5320 mT in 2002 (FEAP, 2003). Growth of turbot is affected by both sex and maturation. Males start to grow less than females as early as 8 months from hatch (Imsland et al., 1997), and this differential growth rate is maintained throughout the remainder of the production cycle including sexual maturation. Maturing females can reach 1.8 kg in 20 months whereas weight of males reaches only around 1 kg. As is practiced with other cultured species, it has been suggested that methods should be developed for the production of all-female populations of turbot (Imsland et al., 1997).

All-female populations of fish can be produced by direct hormonal treatment with estrogens to feminize sexually undifferentiated fish (see Piferrer, 2001 for review). However, despite that steroids are permitted for sex control during early development of fish in the legislation of many countries, this practice provokes consumer rejection and is not advisable. An indirect method based on the production of neomales (genetic females/phenotypic males) can be applied to obtain all-female progenies when the female is the homogametic sex (Piferrer, 2001). To the best of our knowledge, for turbot, there are no available data on hormonal methods, either direct or indirect, to produce all-female populations.

Alternatively, a short-cut approach to obtain all-female populations in fish is through the induction of gynogenesis. Gynogenesis is a chromosome set manipulation technique consisting of the generation of progenies whose chromosomes are exclusively inherited from the mother (Chourrout, 1982; Thorgaard, 1983). The induction of gynogenesis involves DNA sperm inactivation while maintaining its capacity for triggering of embryonic development. The resulting embryos are haploid and nonviable posthatch, unless diploidy is restored by retaining the second polar body or by inhibiting the first mitotic division after shock treatment (Thorgaard, 1983). The induction of gynogenesis results in a low percentage of viable fish because of the manipulations involved and because of high inbreeding, but the resulting fish should be all females when sex determination involves female homogamety (Devlin and Nagahama, 2002). In practice, even in these cases, gynogenesis does not always ensure 100% females, although the offsprings are highly skewed in that direction

(Felip et al., 2001, for review). These deviations can be explained by the influence of the environment or the role of secondary sex determination mechanisms (Komen et al., 1992; Devlin and Nagahama, 2002). Because of the low viability of inbred gynogenetics, a practical approach is to sex-reverse gynogenetics for obtaining neo-males for monosex milt production and to produce all-female progenies (Piferrer et al., 1994; Donaldson, 1996; Felip et al., 2001).

Gynogenesis has other important applications for aquaculture and specifically to that of turbot. First, analysis of sex ratios in gynogenetic progenies can provide valuable data for assessing the sex determination mechanism (Hunter and Donaldson, 1983; Nanda et al., 1992) which is not yet known in the turbot. Although inbreeding decreases viability, highly inbred lines could be crossed to exploit the dominant component of genetic variance (Purdom, 1993; Tave, 1993). Finally, the use of haploid and diploid gynogenetics is broadly recognized as a useful tool for constructing genetic maps (Danzmann and Gharbi, 2001), which now are being implemented in turbot (L. Sánchez, personal communication).

A critical point of gynogenesis induction is the application of the appropriate UV dose to achieve the complete DNA sperm inactivation while maintaining the capacity to trigger embryonic development (Felip et al., 1999). Turbot exhibit poor sperm quality, with considerable variation in concentration among different males (Suquet et al., 1994), and lower larval survival (Devauchelle et al., 1988) when compared to other teleosts. On the other hand, of relevance for this study are the knowledge of turbot sperm physiological features (Suquet et al., 1994), the initiation of movement and swimming characteristics (Chauvaud et al., 1995), and the determination of the optimal sperm-to-egg ratio for fertilization (Suquet et al., 1995; Chereguini et al., 1999). Furthermore, an optimized cold shock procedure to retain the second polar body is available (Piferrer et al., 2000, 2003). The induction of gynogenesis has been reported for other flatfishes including the hirame, *Paralichthys olivaceus* (Tabata, 1991; Kim et al., 1993; Yamamoto, 1999) and the common sole, *Solea solea* (Howell et al., 1995). Currently, gynogenesis is used in the practical aquaculture of rainbow (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) in France, common carp (*Cyprinus carpio*) in China and Japan and of hirame in Japan (Hulata, 2001).

The objectives of the present study were: (1) to investigate the effects of UV light on turbot sperm in regard to its ability to fertilize (activate) eggs and trigger embryonic development, (2) to determine the optimal conditions to induce gynogenesis in the turbot, and (3) to study early development and viability of gynogenetic progeny.

2. Materials and methods

2.1. Gamete collection and artificial fertilization

Turbot broodstock reared at the facilities of the Centro Oceanógrafico de Vigo (NW Spain) were switched to a constant photoperiod of 16 h of light:8 h of darkness, and a constant water temperature of 13–14 °C 60 days before use to stimulate natural maturation. Eggs from ovulated females and milt from running males were obtained

during March–June by abdominal massage. Egg quality (egg diameter ~ 1.1 mm; 1 ml of eggs ~ 800 eggs) was assessed according to the criteria of McEvoy (1984). Artificial fertilization was performed according to procedures described in Piferrer et al. (2000, 2003). No attempts were made to separate viable and nonviable eggs. Viability was assessed in a sample of fertilized eggs by placing them in a graduated cylinder and allowing them to sit for about 5 min after which the floating proportion was measured. As a precaution, egg batches with less than 50% survival 24 h after fertilization were discarded.

Induction of gynogenesis was carried out by fertilizing the eggs with sperm whose DNA had been previously irradiated with UV light. The diluent used was Ringer-200, pH 8.1 (Chereguini et al., 1997). The UV source was four G15T8 15-W UV lamps with maximum emission at 254 nm (Sylvania) placed ~ 30 cm above the sperm layer (~ 0.3 mm thick) in a Petri dish on top of crushed ice. The desired irradiation dose was achieved by modifying exposure duration. The motility of the irradiated sperm was microscopically checked by estimating the amount of motile spermatozoa and the duration of motility after its activation with seawater (Suquet et al., 1992; Chereguini et al., 1999). Diploidy was restored by applying a thermal shock treatment to the eggs shortly after fertilization (Piferrer et al., 2000, 2003). Control and treated groups were incubated in Plexiglas cylinders (15 cm diameter, 3-l capacity), fitted with a bottom mesh (300 μm pore) partially submerged inside a tank provided with recirculated, filtered, UV-sterilized and aerated seawater, thermoregulated at 13–14 °C. To achieve optimum induction of gynogenesis, four experiments were designed:

2.2. Experiment 1. Effects of dilution on fertilization capacity and UV irradiation on sperm motility

Experiment 1a examined the variation in sperm quality due to source (male donor) and to dilution for their effects on egg fertilization capacity (no UV irradiation was involved). Conversely, Experiments 1b and 1c did not involve the use of eggs or fertilizations but instead explored the effects of UV irradiation on sperm motility. Experiment 1b tested the influence of three different sperm dilutions: 1:5, 1:10 and 1:20 (to allow different penetration capacities) on the effects of UV irradiation on sperm motility. Finally, Experiment 1c was performed with sperm dilution set at 1:10 and determined the effect of UV irradiation on sperm motility, considering both score (amount) and duration.

2.3. Experiment 2. The Hertwig effect

Based on the results from Experiment 1, in Experiment 2, we determined the dose of UV irradiation necessary for full inactivation of sperm DNA without compromising its capacity to activate embryonic development; that is, the dose at which the Hertwig effect is elicited (Thorgaard, 1983). Aliquots of eggs were activated with sperm diluted 1:10 and irradiated with UV light at increasing intensities from 300 to 100,000 erg mm^{-2}. Survival and external morphology of the embryos and larvae were determined at 4.5, 72 and 144 h postfertilization (hpf).

2.4. Experiment 3. Low-scale production of gynogenetic turbot

Experiment 3 was carried out to induce gynogenesis. The eggs were activated with sperm diluted 1:10 and irradiated with 30,000 erg mm^{-2} of UV light, as determined in Experiment 2, and were held in water at 13 °C. A cold shock treatment was applied to retain the second polar body for restoring diploidy by transferring the eggs to water at −1 to 0 °C for 25 min, starting at 6.5 min after activation (Piferrer et al., 2003). Fertilization, embryogenesis and hatching rates were determined at 4.5, 72 and 144 hpf, respectively.

2.5. Experiment 4. Large-scale production of gynogenetic turbot

Eggs were obtained from one female and were divided approximately into two equal batches. Sperms were obtained from one male and were divided into two unequal aliquots (details in Table 1). One batch was fertilized with diluted sperm not exposed to UV irradiation and was used as the diploid control, whereas the other batch was activated with diluted, UV-irradiated sperm and was cold shocked to induce gynogenesis according to the conditions established in Experiment 3. Fish were reared using standard protocols for turbot. Survival was determined at 1, 22 and 180 days posthatch (dph). In the last sampling, growth (weight and length) was also determined. The entire experiment was repeated a second time.

2.6. Survival and ploidy determination

Under the incubation conditions described above, hatching typically took place at 5 dpf and lasted 1 day. Survival was calculated as described in Piferrer et al. (2000); the

Table 1
Induction of gynogenesis in turbot on a large scale (Experiment 4)

Variable	Control diploids	Gynogenetic diploids	Significance level
Sperm concentration (spz/ml)	$1.4 \pm 0.16 \times 10^9$	$1.4 \pm 0.16 \times 10^9$	N/A
Volume of eggs used (ml)	42–47	55–62	N/A
Approximate total number of eggs used	$33.6–37.6 \times 10^3$	$44.0–49.6 \times 10^3$	N/A
Volume (ml) of sperm used after being diluted 1:10	1	4 (UV-irradiated)	N/A
Fertilization (%)	92.5 ± 0.6	10.8 ± 2.2	$P<0.01$
Survival (%) at 1 dph with respect to total number of eggs used	22.3 ± 0.4	4.5 ± 0.9	$P<0.01$
Survival (%) in the period 1–22 dph	16.1 ± 9.8	8.9 ± 6.7	NS
Survival (%) in the period from 22 dph to 6 months	95.4	86.8	N/A
Weight at 6 months (g)	118.8 ± 3.9 ($n=33$)	112.0 ± 4.0 ($n=33$)	NS
Total length at 6 months (cm)	16.8 ± 0.2 ($n=33$)	16.7 ± 0.2 ($n=33$)	NS

Fertilization characteristics, survival and growth, up to 6 months of age, of gynogenetic diploid turbot as compared to control diploids.
Notes: spz, spermatozoa; dph, days posthatch; N/A, does not apply; NS, not significant. Data as mean ± S.E.M. of two separate experiments.

nonfertilized eggs, nonhatched eggs and the larvae were counted and were added to obtain the total number of eggs initially used in each group. Survival was calculated 1 dph as the number of live larvae, with respect to the number of initial eggs, and was expressed as a percentage.

Ploidy was determined in larvae of Experiments 3 and 4 collected 1 dph, except for the UV-irradiated groups, where embryos were used because of the nonviability of haploid larvae. Ploidy determination was evaluated by counting the number of nucleolar organizing regions (NOR) and by direct counting of the number of chromosomes in a subset of larvae in each group (Piferrer et al., 2000). In Experiment 4, the gynogenetic nature of the fish produced by UV-irradiated sperm followed by cold shock was also verified in a sample of 20 larvae subjected to analysis with microsatellite DNA markers developed for the turbot (Castro et al., 2003).

2.7. Statistical analysis of data

Only trials in which actual survival 1 dph in controls was >30% were used. Thus, the data presented were obtained from separate trials with eggs from different females. Survival at 1 dph was transformed to percentages and was expressed, relative to the survival of the untreated control which was set at 100% (Volckaert et al., 1994; Felip et al., 1999). Percentage data were arcsin transformed before analysis of variance (ANOVA). Analyses were followed by Tukey's Honest Significant Differences test (Sokal and Rohlf, 1995). Data are expressed as mean ± S.E.M. Differences were accepted as significant when $P < 0.05$.

3. Results

3.1. Preliminary trials

Turbot sperm concentration varies greatly among males and its quality is poor when compared to that of other teleosts (Suquet et al., 1994). These circumstances led us to attempt to standardize sperm concentration through dilution prior to UV irradiation. Preliminary attempts failed because sperm obtained from different males and diluted to the same final concentration responded quite differently to the effects of UV irradiation (data not shown). When using sperm samples (1:10 dilution) from several males, it was then found that there was no significant ($r^2 = 0.17$; $P > 0.05$) relationship between predilution sperm concentration and the UV dose at which the amount of motile sperm is reduced to half of the initial value (ID_{50}). For example, four males whose sperm concentration was quite similar and just above 6×10^9 spermatozoa ml^{-1} had quite different ID_{50} values (Fig. 1).

3.2. Experiment 1a

To test differences in sperm quality and the effect of dilution, the sperm of four different males was diluted each from 1:10 to 1:100 and was used to fertilize aliquots

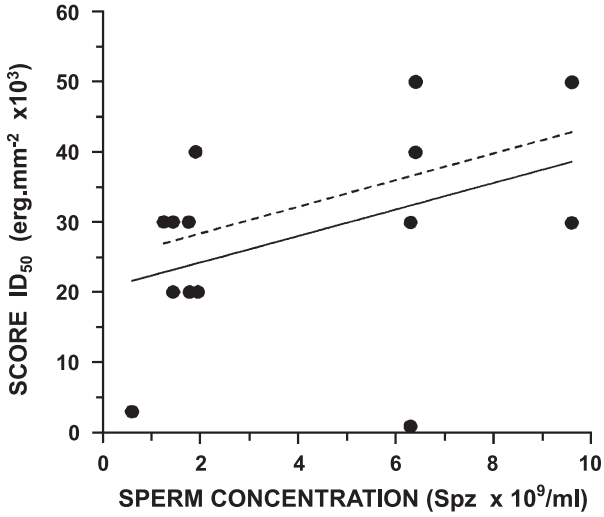

Fig. 1. Lack of relationship between turbot sperm predilution concentration and motility score ID_{50} for UV-irradiated sperm (preliminary trials). Sperm was diluted 1:10 with Ringer-200 prior to UV irradiation. Each datapoint (●) is the value corresponding to sperm from different males ($n = 14$). Lines only indicate correlation tendencies because the relationship between sperm concentration and score ID_{50} was not statistically significant ($P > 0.05$). (—) All datapoints considered; (- - -) the two males with $ID_{50} < 5 \times 10^3$ erg mm^{-2} were not included.

of the same pool of eggs, achieving different sperm/egg ratios. Results showed that percent fertilization is more related to male than to dilution (Fig. 2). Furthermore, while the sperm of three out of four males tested gave fertilizations of ~80% or

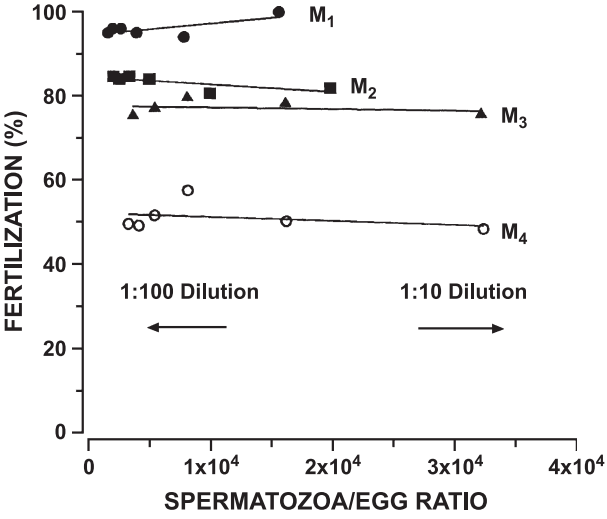

Fig. 2. Effect of the spermatozoa/egg ratio on the fertilization rate in turbot according to donor male and sperm dilution (Experiment 1a). Four different males [M_1 (●), M_2 (■), M_3 (▲), M_4 (○)] were used, and the sperm of each was diluted at six different dilutions, from 1:10 to 1:100.

higher, the sperm of the remaining male (M_4) gave fertilizations of ~ 50% regardless of dilution.

3.3. Experiment 1b

Tests carried out with the sperm from a single male each time and subjected to different dilutions (1:5, 1:10 or 1:20) showed no differences between the 1:5 and 1:10 dilutions in the effect of increasing UV doses on motility duration. However, when the sperm was diluted 1:20, the motility duration was greatly compromised, indicating stronger effects of UV light because of easier penetration due to dilution (Fig. 3). Together, results obtained so far indicate that it was not worth adjusting sperm concentration prior to UV irradiation and that 1:10 was a good dilution to irradiate turbot sperm under the conditions employed.

3.4. Experiment 1c

The effects of UV irradiation on sperm motility were assessed using individual sperm samples obtained from nine different males. Results show that as the irradiation dose increased, there was a decrease in sperm motility, both in the percentage of motile spermatozoa, referred to as motility score (Fig. 4A), as well as in the motility duration (Fig. 4B). A semilogarithmic representation of data evidenced a typical dose–response relationship with a similar ID_{50} value in both the motility score (Fig. 4A) and motility duration (Fig. 4B) ($28.1 \pm 3.9 \times 10^3$ vs. $28.7 \pm 6.6 \times 10^3$ erg mm^{-2}, respectively).

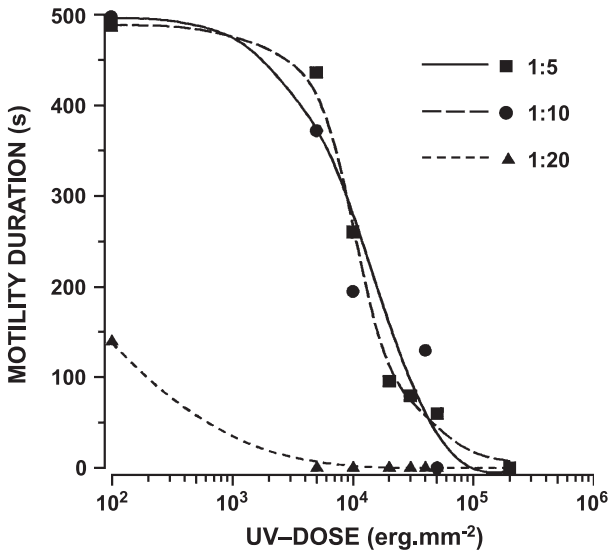

Fig. 3. Effect of exposure to different doses of UV light on the duration of the motility of turbot spermatozoa subjected to different dilutions [1:5 (■), 1:10 (●) or 1:20 (▲)] with Ringer-200 (Experiment 1b). Data are from one male and are representative of three separate replications.

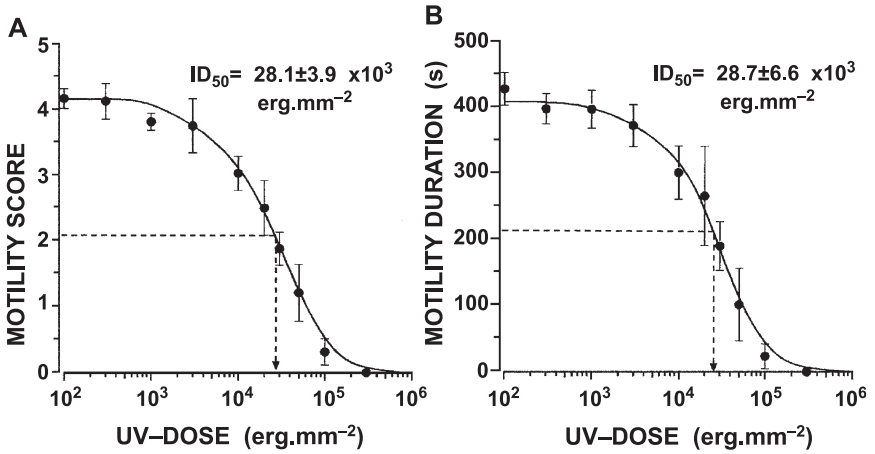

Fig. 4. Effect of exposure to different doses of UV light on the motility of turbot spermatozoa diluted 1:10 with Ringer-200 (Experiment 1c). (A) Effect on the motility score, classes 0 to 5, according to Chereguini et al. (1997). (B) Effect on motility duration. The ID_{50} was calculated as the dose in which the motility score or duration was reduced to 50% with respect to the original value. Data as mean ± S.E.M. of nine separate experiments, each with the sperm of a single male.

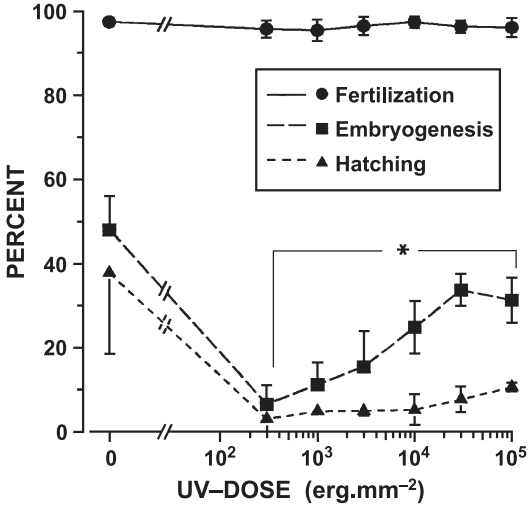

Fig. 5. Effect of exposure to different doses of UV light on the survival of turbot at three different times during early ontogenesis: 4.5-h postfertilization (hpf) = "Fertilization (●)"; 72-hpf = "Embryogenesis (■)"; and 144 hpf (equivalent to 1 dph) = "Hatching (▲)" (Experiment 2). Sperm was diluted 1:10 with Ringer-200 prior to UV irradiation. A typical Hertwig effect took place between 300 and 30,000 erg mm^{-2}. Each datapoint is the mean ± S.E.M. of three separate experiments. (*) Statistically significant (ANOVA; $P < 0.05$) increase in survival at 72 hpf within the marked dose range with respect to the 3×10^2 erg mm^{-2} dose.

3.5. Experiment 2

Eggs of a single female were activated with aliquots of sperm from a single male after being irradiated at different doses. Activation rates were not affected but significant differences in embryogenesis were detected among increasing UV doses (ANOVA, $P<0.05$). Embryogenesis decreased at 300 erg mm^{-2} but a continuous increase in the number of embryos at 72 hpf was seen up to 30,000 erg mm^{-2} (Fig. 5). These results are typical of the Hertwig effect, and for this reason, 30,000 erg mm^{-2} was considered the appropriate dose of UV light to inactivate the turbot sperm while maintaining their capacity to activate embryo development.

3.6. Experiment 3

Gynogenesis induction in the turbot was achieved by fertilizing eggs with UV-irradiated (30,000 erg mm^{-2}) sperm (diluted 1:10) followed by a cold shock at -1 to 0°C for 25 min, starting at 6.5 min after fertilization. The survival of gynogenetic diploid turbot was significantly lower ($P<0.05$) than that of the untreated diploid controls in all the three developmental stages examined (Fig. 6). In addition, the

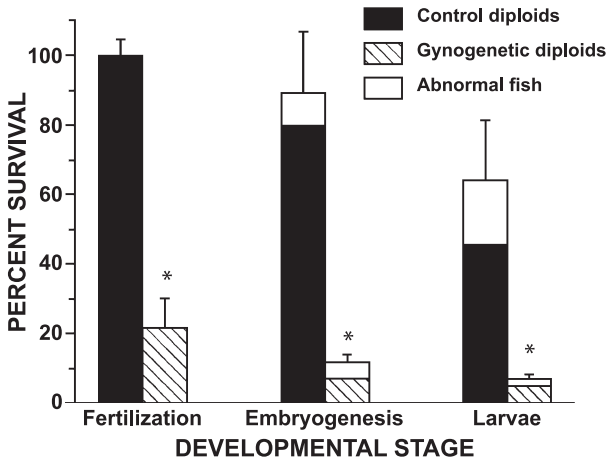

Fig. 6. Effect of gynogenesis induction on the viability of turbot during the early developmental stages: 4.5-h postfertilization (hpf) = "Fertilization"; 72-hpf = "Embryogenesis"; and 144 hpf (equivalent to 1 day posthatching) = "Hatching". (Solid bars) Diploid control group made with sperm diluted to 1:10 (control of gamete quality, with survival at fertilization set to 100% to which the other survival data was compared). Actual survival of the diploid control at fertilization was 44.3 ± 16.4%. (Hatched bars) Gynogenetic diploid group produced with sperm diluted 1:10, irradiated with 30,000 erg mm^{-2} and a thermal shock of the activated eggs (-1 to 0°C, applied for 25 min starting at 6.5-min postfertilization) to restore diploidy (effect of UV light + thermal shock; Experiment 3). Data are as mean ± S.E.M. of three separate experiments, with duplicate determinations for each datapoint. (Bars) Survival, with the shaded part referring to the proportion of normal fish and the white part to fish with morphological abnormalities. (*) Significant differences ($P<0.05$) in survival between control diploids and gynogenetic diploids within the same developmental stage. No significant differences were found in survival among different developmental stages within each group.

percentage of embryos with abnormalities was higher in the gynogenetics (Fig. 6). Embryos (Fig. 7A) and hatched larvae (Fig. 7B) from the diploid control had a morphologic normal appearance while embryos originated from eggs activated with sperm exposed to 30,000 erg mm^{-2} of UV light and non cold-shocked exhibited aberrant development (Fig. 7C). The few larvae that hatched were deformed, exhibiting

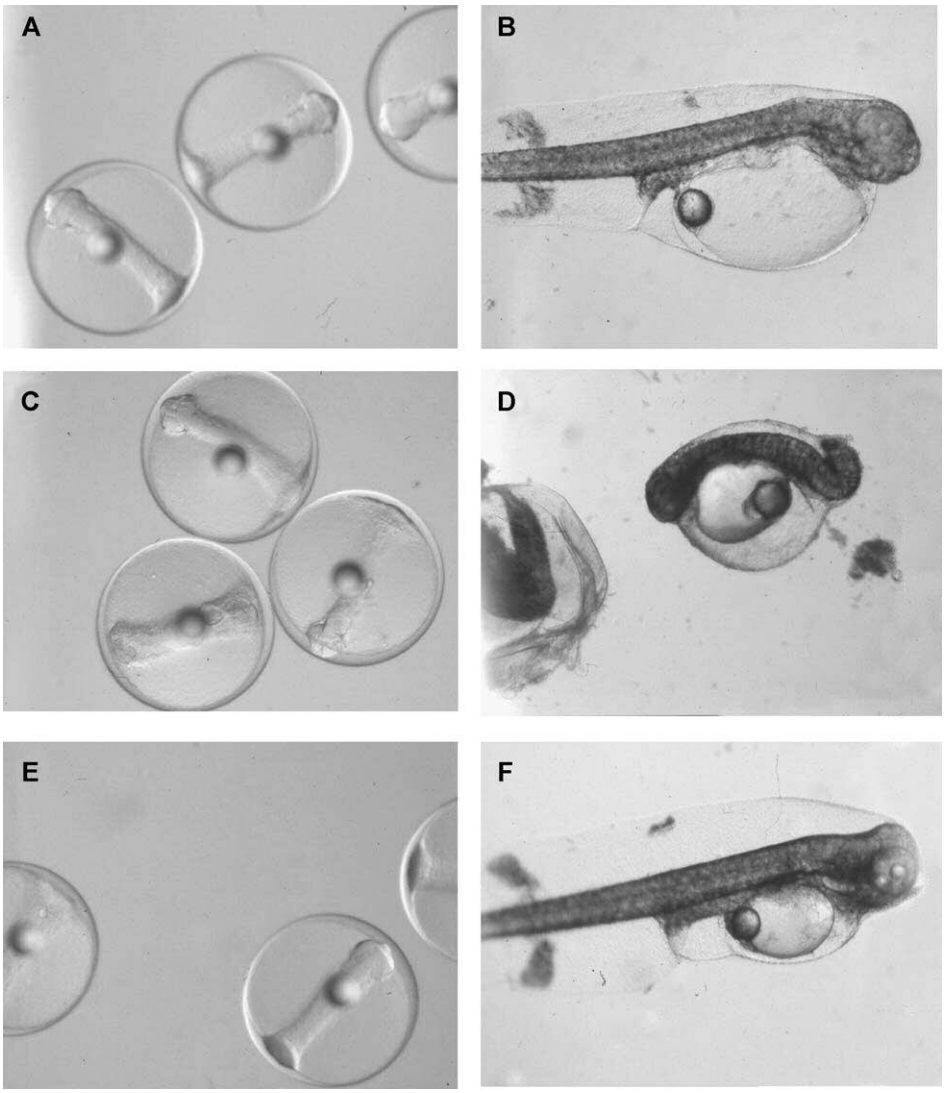

Fig. 7. External appearance of turbot embryos 72 h after fertilization (left panels) and larvae at 1 day after hatching (right panels). (A and B) Eggs fertilized with nonirradiated sperm (control diploids); (C and D) Eggs activated with UV-irradiated sperm (haploids); (E and F) Eggs activated with UV-irradiated sperm and cold shocked (gynogenetic diploids). Note the "haploid syndrome" in panel D.

a typical "haploid syndrome" (Fig. 7D), thus indicating that these fish were haploids. Haploids did not survive for more than 1 day. In contrast, diploid gynogenetic eggs (Fig. 7E) and larvae (Fig. 7F) had normal appearance, similar to that of diploid controls.

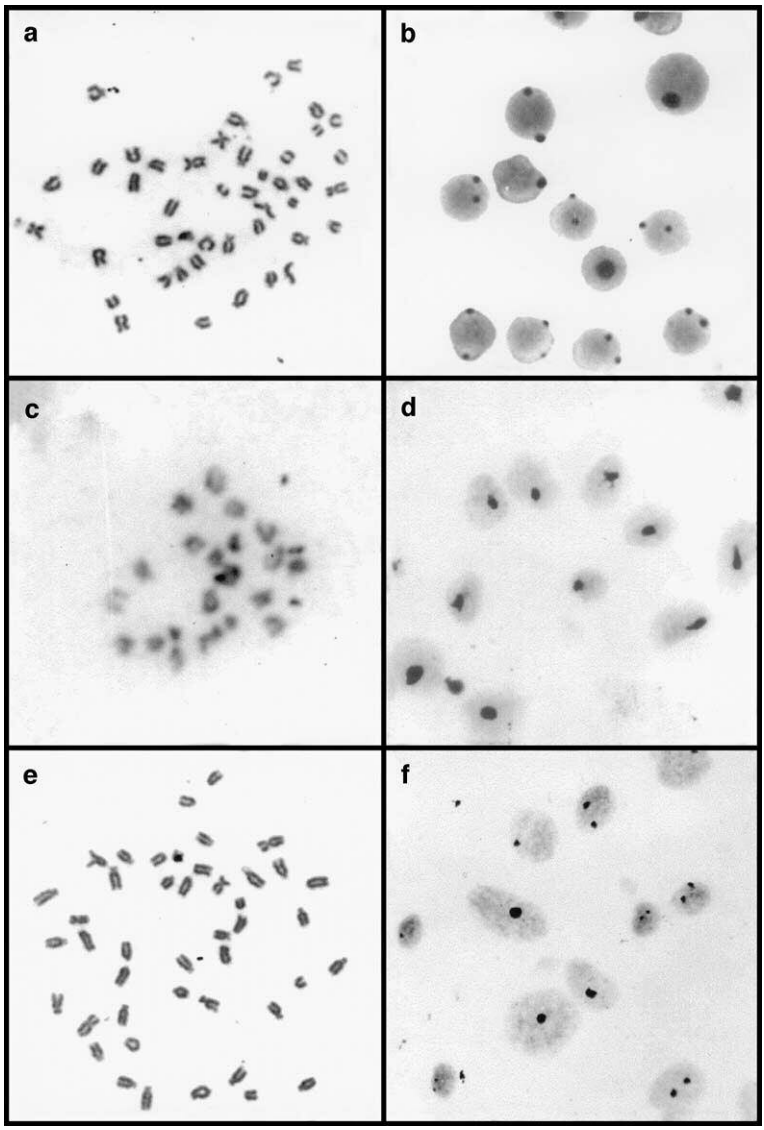

Fig. 8. Ploidy identification in turbot embryos 72 h after fertilization (left panels) and larvae 1 day after hatching (right panels). Typical metaphase spreads, and Ag-stained nuclei of cells obtained from control diploids (a and b, respectively; $2n = 44$), gynogenetic haploids (c and d, respectively; $n = 22$) and gynogenetic diploids (e and f, respectively; $2n = 44$).

Embryos and larvae of the control diploid group had cells containing 44 chromosomes (Fig. 8a) and one or two nucleoli (Fig. 8b) as expected. In contrast, embryos and larvae resulting from the UV-irradiated group not cold shocked had cells with 22 chromosomes (Fig. 8c) and only one nucleolus per nucleus (Fig. 8d). In the group activated with UV-irradiated sperm and cold shocked, diploidy and viability were restored. The cells of the fish from this group had the standard turbot karyotype of 44 chromosomes (Fig. 8e) and one or two nucleoli per nucleus (Fig. 8f), indicating that they were gynogenetic diploids. Sometimes, aneuploid metaphases were observed, but in all cases the modal number of chromosomes matched the expected ploidy level.

3.7. Experiment 4

The results of the induction of gynogenesis using a large volume of turbot eggs are presented in Table 1. This experiment was repeated twice, using the eggs and sperm of two females and males in each, creating two diploid control and two gynogenetic diploid groups. The induction of gynogenesis significantly reduced ($P < 0.01$) both the activation rate and survival at 1 dph, in accordance with earlier observations (Fig. 6). Microsatellite analysis verified that each one of the 20 analyzed larvae in the two UV-irradiated and cold-shocked groups had only maternally derived DNA (Castro et al., 2003). Thus, the induction of gynogenesis was 100% in both families. Although survival in the period 1–22 dph was reduced approximately by half in the gynogenetics as compared to controls, no statistically significant differences were detected due to variation between the two families. Thereafter, survival to 180 dph (6 months) was similar between controls (95.4%) and gynogenetics (86.8%). At 6 months, the gynogenetics had grown to over 100 g in weight and ~ 17 cm in total length (TL) in a manner similar to that of the controls, exhibiting no statistically significant differences in these variables (Table 1).

4. Discussion

In this study, a protocol to produce gynogenetic turbot was developed involving a combination of UV irradiation of the sperm, followed by the application of a cold shock to the newly activated eggs. The effective dose of UV light to completely inactivate sperm DNA while maintaining its activation ability was 30,000 erg mm^{-2}. These results are similar to other previously reported to elicit the Hertwig effect (Felip et al., 2001), suggesting a conserved dose–effect relationship among different marine fish species. In addition, this dose was also very close to the ID_{50} (~ 28,000 erg mm^{-2}) on sperm motility, also determined in this study. Thus, the necessary dose of UV light required for inactivation of sperm DNA results in a reduction of the motile score from approximately 4 to 2, implying that about 25% of the spermatozoa (spz) remained motile after exposure to UV light. Therefore, starting from a typical sperm concentration of $2–4 \times 10^9$ spz/ml (turbot range of $0.7–11 \times 10^9$ spz/ml; Fauvel et al., 1993) and accounting for the dilution of 1:10 used, it follows that at least 50×10^6 spz/ml were available for fertilization. With this dose, sperm motility duration was reduced from ~ 400 s (~ 6 min) to ~ 200 s (~ 3 min), a time well within the range (1–17 min) required for sperm–egg contact during

artificial fertilization of the turbot, as determined by Suquet et al. (1994). In addition, the effective dose of 30,000 erg mm^{-2} did not result in a significant ($P>0.05$) decline in the number of live embryos at 48 hpf when compared to the nonirradiated controls (34% vs. 48%; Fig. 5).

It is well known the low power of penetration of UV light and hence the dependence of the response to increasing doses of UV light on sperm dilution (Häder, 1993). As expected, the higher the dilution, the easier UV light could penetrate and exert its effects, as observed in our study in individual trials. However, the lack of relationship between initial sperm concentration and response to UV light when several males were evaluated indicates that there are other factors related to sperm "quality" more important than its concentration in determining the individual response to UV light. In the test using sperm from different males, even with the highest dilutions, the motile spermatozoa-to-egg ratio still was within the optimum range of 3000–6000 suggested for an optimal fertilization in turbot (Suquet et al., 1995; Chereguini et al., 1999). Thus, it appears that, at least in the turbot, the lower viability of gynogenetics cannot be due to lower fertilization rate because of lower number of motile spermatozoa, as suggested by Felip et al. (1999) for the sea bass. Nevertheless, in the mass production of gynogenetic diploids, survival of these fish was about 1/10 of the controls. Furthermore, it was observed that the amount of larvae with any sort of external abnormalities in the control diploids represented about one third of the total larvae. When methods for chromosome set manipulation are scaled-up to a semiindustrial or industrial level, a reduction of the yield is usually accompanied by an increase in mortality and in abnormal fish (Felip et al., 1999). This may be due to the increased mechanical stress produced by the handling of a considerable amount of eggs.

Preliminary assays of sperm inactivation for obtaining gynogenetic turbot had been carried out by Vázquez et al. (2000, 2002). In these assays, sperm dilution was 1:9 and the irradiation procedure was similar to that used in this study. However, it was concluded that the best UV dose was 87×10^3 erg mm^{-2}. In view of our results, this dose seems too high, which would explain the low fertilization and survival observed in the study by Vázquez et al. (2002). Eliciting a proper Hertwig effect is important because it allows finding the dose that ensures sperm inactivation (by changing conformation of DNA), although results may be slightly different whether UV or gamma irradiation is used (Chourrout et al., 1980). Lower UV doses result in aneuploid embryos with very low survival during embryogenesis, while doses above the optimal dose for the Hertwig effect (>30,000 erg mm^{-2} in our case) can provoke further damage (e.g., chromosome fragmentation) resulting in $<1n$ embryos. This was probably the situation found by Vázquez et al. (2000, 2002) which would account for the extremely low viability recorded.

Gynogenetic fish were initially determined by direct chromosome number count and NOR analysis, as previously performed to identify triploid turbot (Piferrer et al., 2000). However, the need to obtain metaphase spreads from solid tissues in small embryos (Kligerman and Bloom, 1977) and the existence of a low intensity NOR–number polymorphism in turbot (Pardo et al., 2001) compromised the efficiency of using this technique to verify gynogenesis. Therefore, the true maternal inheritance of the families was verified by using microsatellite DNA markers (Castro et al., 2003) and was found that the putative gynogenetic groups were in fact 100% gynogenetics.

Turbot has little or no influence from the environment on the proportion of sexes because under a variety of culture conditions, sex ratios do not differ from 1:1 male/female, suggesting a simple chromosomal system of sex determination. If this species turns out to have female homogamety, as it has been reported for several species of pleuronectiformes (Devlin and Nagahama, 2002), the induction of gynogenesis not only will help to discern the sex-determining mechanism of turbot but also could be a way for producing all-female populations based on the production of neomales from gynogenetic diploids. Furthermore, gynogenetics constitute a very valuable tool for other areas of research related with culture improvement in turbot like the enhancement of production through heterosis (Purdom, 1976). In addition, the availability of haploid and diploid gynogenetics represents a useful material for obtaining refined genetic maps for different genetic markers including distances between these markers and centromeres.

In conclusion, this paper reports the effects of UV irradiation of sperm in the turbot, a species characterized by a low sperm count and concentration, and provides a method for the induction of gynogenesis at an industrial scale. The survival, growth and reproduction of adult gynogenetic diploid turbot, with specific emphasis on gonadal morphology, histology and sex ratios, will be reported elsewhere.

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Artículo 6

Gynogenesis Assessment Using Microsatellite Genetic Markers in Turbot (*Scophthalmus maximus*)

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Gynogenesis Assessment Using Microsatellite Genetic Markers in Turbot (*Scophthalmus maximus*)

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Abstract: Gynogenesis was assessed by different methods in 2 families of gynogenetic offspring in turbot (*Scophthalmus maximus*). Karyotype analysis in embryos and larvae demonstrated high accuracy in estimation of ploidy level, but performance was uneven given the low quality and number of plates obtained. The use of silver staining to estimate the number of nucleoli per nucleus resulted in a straightforward and easy method to evaluate the ploidy of the samples studied. However, the existence of a nucleolus organizer region polymorphism in turbot determined a small error in ploidy estimation, important when checking ploidy in specific individuals. The use of a set of 11 highly variable microsatellite loci proved to be a powerful method to confirm the exclusive maternal inheritance to gynogenetic offspring in turbot, with probabilities of detection of putative paternal genetic contribution above 99.99%.

Keywords: gynogenesis, microsatellites, turbot, nucleolus organizer regions.

INTRODUCTION

Production of all-female or sterile populations by chromosome set manipulation has been broadly applied during the last decades to improve fish culture (Benfey, 1989; Ihssen et al., 1990). Under intensive farming conditions females grow markedly faster than males in many fish species (Piferrer, 2001). In addition, sexual maturation usually involves a reduction in growth rate and increased mortality in fish. In order to overcome these undesirable effects, gynogenesis and triploidy have been induced in many fish species (Thorgaard, 1995; Donaldson et al., 1996; Felip et al., 2001). Since the pioneering studies of Purdom (1972) with plaice, chromosome set manipulation has been performed in several marine fish of great importance for aquaculture such as the sea bream *Sparus aurata* (Gorshkova et al., 1995; Garrido-Ramos et al., 1996), the sea bass *Dicentrarchus labrax* (Gorshkova et al., 1995; Felip et al., 1997), and some flatfish including the common sole *Solea solea* (Howell et al., 1995), the halibut *Hippoglossus hippoglossus* (Holmefjord and Refstie, 1997), and the turbot *Scophthalmus maximus* (Piferrer et al., 2000, 2003).

Although occurring naturally in some fish species, gynogenesis is usually achieved by fertilization of eggs with genetically inactivated sperm using radiation or chemical treatments, or with heterologous sperm that triggers development without any genetic contribution to the egg (Thorgaard, 1983; Ihssen et al., 1990; Felip et al., 2001). In

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this way, the genome of the resulting haploid zygote is exclusively of maternal origin. As the haploid embryos are usually not viable, the diploid condition is restored by retention of the second polar body or prevention of the first mitotic division by means of pressure or thermal treatments (Chourrout, 1982; Thorgaard, 1983; Felip et al., 2001). A reliable method to confirm the gynogenetic nature of the offspring obtained is essential to go forward with possible applications of this method to fish culture.

Determination of the ploidy level has been the usual approach for assessing gynogenetic progenies. In this sense, karyologic analysis allows direct confirmation of the ploidy when good metaphase plates are available, a challenge in many fish species (Chourrout and Quillet, 1982; Piferrer et al., 2000). A more straightforward and extensively applied technique is the estimation of the number of nucleoli per cell by silver staining, a technique commonly used to detect nucleolus organizer regions (NORs; Howell and Black, 1980; Hubbell, 1995). Assuming a single NOR locus in the karyotype of the species studied, a maximum of 1, 2, and 3 nucleoli per cell should be detected in haploid, diploid, and triploid cells, respectively (Phillips et al., 1989; Felip et al., 1997; Piferrer et al., 2000). However, the existence of polymorphism in the number of NORs or multichromosomal location of NORs in the species of interest would not accord with this assumption. This fact could determine the existence of more than 1, 2, and 3 nucleoli in haploid, diploid, and triploid individuals, respectively, and also the overlapping of the mean number of nucleoli per nucleus distributions in individuals of different ploidies, therefore decreasing the accuracy of ploidy determination (Phillips et al., 1989; Castro et al., 1996; Piferrer et al., 2000; Pardo et al., 2001).

Although routinely used these methods do not confirm per se the exclusive maternal transmission to offspring. The use of biochemical markers, like allozyme loci, has allowed the unambiguous monitoring of maternal inheritance in some fish species (Yousefian et al., 1996). However, the low genetic variability usually associated with these markers has limited their potential use. The development of highly variable genetic markers in the last years has provided the source of polymorphism needed to achieve genetic identification in fish. Parentage and pedigree analysis is currently available in many fish species by using DNA markers like minisatellites and microsatellites (O'Reilly and Wright, 1995; Carvalho and Hauser, 1998; Ferguson and Danzmann, 1998). The application of DNA genetic markers to verify gynogenesis in fish seems promising. Felip et al. (2000) have recently demonstrated uniparental inheritance in the sea bass by using amplified fragment length polymorphism (AFLP).

The turbot *Scophthalmus maximus* is a commercially valuable flatfish species, whose farming production has experienced an increasing demand in the last decade. As in other fish, growth rates are markedly higher in turbot females than in males. Sexual maturation can take place before reaching marketable size, involves a reduction in somatic growth, and increases mortality. Production of all-female or sterile populations by chromosome set manipulation is, therefore, a matter of interest in turbot culture (Piferrer et al., 2000). In this paper we have analyzed the potential of a set of microsatellite loci for parentage assignment and their usefulness for checking the maternal inheritance of gynogenetic offspring in turbot.

Materials and Methods

Induction of Gynogenesis in Turbot

Gynogenetic turbot were produced at the facilities of the Instituto Español de Oceanografia de Vigo (I.E.O) in Spain, in 2000 and 2001. Two crosses were performed using eggs and sperm from a single female and male, in each case. Gametes were treated according to the method of Cal et al. (2002) to obtain gynogenetic turbot. Eggs from each female were divided in 3 batches. To obtain diploid gynogenetics, one batch was fertilized with UV-irradiated sperm followed by a cold shock between 0° and −1°C for 25 minutes, starting 6.5 minutes after fertilization (Piferrer et al., 2002). The other 2 batches were used as controls to check the inactivation of sperm following UV irradiation (haploid gynogenetics) and the cold shock applied for retention of the second polar body (triploids).

Ploidy Determination

Ploidy was examined in larvae collected 1 day after hatching, except for the sperm-irradiated control, where embryos were used because of the nonviability of haploid larvae. Samples were maintained in a solution of 0.005% colchicine for 6 hours. Metaphase spreads were obtained according to the method of Kligerman and Bloom (1977). Slides were stained with silver nitrate as described in Howell and Black (1980) and counterstained with 3% Giemsa during 3 minutes to reveal NOR regions and nucleoli. The

number of nucleoli per nucleus was determined in about 50 nuclei per larva or embryo. Twenty individuals were analyzed per group in each experiment. When possible (usually in around 5 fishes per group), ploidy was confirmed by counting the number of chromosomes in at least 10 plates per individual.

Microsatellite Analysis

DNA was extracted from fin samples obtained from both parents in each cross, and from whole fish in the progenies (embryos and larvae) using Chelex 100 chelatting resin according to Walsh et al. (1991). Polymerase chain reaction (PCR) was carried out with specific primers designed to amplify 11 previously characterized turbot microsatellites (Coughlan et al., 1996; Estoup et al., 1998; Bouza et al., 2002). PCR conditions used in this work are described in detail in Bouza et al. (2002).

According to the microsatellite genotypes of both parents in each cross performed, 2 diagnostic loci (no alleles shared by both parents) were selected among the 11 analyzed and amplified in 15 to 20 putative diploid gynogenetics in each family, to monitor exclusive maternal inheritance.

The potential of the microsatellite loci for monitoring the production of gynogenetic turbot was evaluated by computing the 2 cumulative exclusion probabilities in an Atlantic population of turbot (Bouza et al., 2002) with the CERVUS 2.0 package (Marshall et al., 1998). Following the recommendations of Queller and Goodnight (1989), this population was selected as a reference to obtain the potential of excluding microsatellites because the broodstock from I.E.O. was founded with native individuals from this area. Exclusion probabilities estimate the power at each locus and the combined probability over loci to exclude a candidate parent from a single individual either knowing only the genotype of this offspring (Excl 1), or also knowing the genotype of one parent (Excl 2).

RESULTS

Karyologic Analysis

Karyologic examinations were limited to a few fishes within each group (gynogenetics and controls) originating from both crosses, especially in the case of embryos. Metaphase spreads could not be obtained from all individuals, and their quality was far from the best in some cases. All chromosome spreads showed the expected ploidy level in most plates analyzed both in the gynogenetic (2n) and control groups (n and 3n, respectively; Figure 1). Aneuploid metaphases were sometimes observed, probably resulting from technical devices, but in all specimens studied the modal number of chromosomes was in accordance with their expected ploidy level. Metaphases of embryos from eggs fertilized with irradiated sperm were haploid (22 chromosomes). Putative gynogenetic diploids showed the standard turbot karyotype (2n = 44 chromosomes). Larvae from eggs fertilized with normal sperm and subjected to cold shock exhibited metaphases with 66 chromosomes as expected in triploids.

Silver Staining

Table 1 shows the average number of nucleoli per nucleus in the fishes analyzed from each group (n, 2n, and 3n) in the 2 crosses performed. In the first cross the mean number of nucleoli ranged from 1.03 in haploids to 2.01 in triploids and was 1.44 for gynogenetic diploids. No range overlapping was observed between the 3 distributions, and each individual could be unambiguously assigned to its ploidy group in this cross. In the second cross, the figures obtained were 1.33, 1.56, and 1.95, respectively. In this case a considerable number of haploid and diploid fishes showed 2 and 3 nucleoli, respectively, which would not be expected for a single NOR locus. Also, the mean number of nucleoli per cell in the gynogenetic haploids and diploids was higher in this cross than in the former, with both distributions slightly overlapping.

Microsatellite Analysis

Eleven microsatellite loci were amplified in the 4 parents used in the 2 crosses performed. Also, a sample of 46 turbot from the Atlantic area in northwestern Spain (Bouza et al., 2002) was used as a reference to estimate the potential of these loci for parentage assignment. Genetic diversity figures of the 11 loci analyzed in the present work are shown in Table 2. The number of alleles ranged from 7 to 16 (locus *Smax-03* and *Sma3-129INRA*, respectively) with a mean figure of 11.55. With the exception of *Smax-03*, all loci showed values of expected heterozygosity (H_e) and polymorphic information content (PIC) between 0.70 and

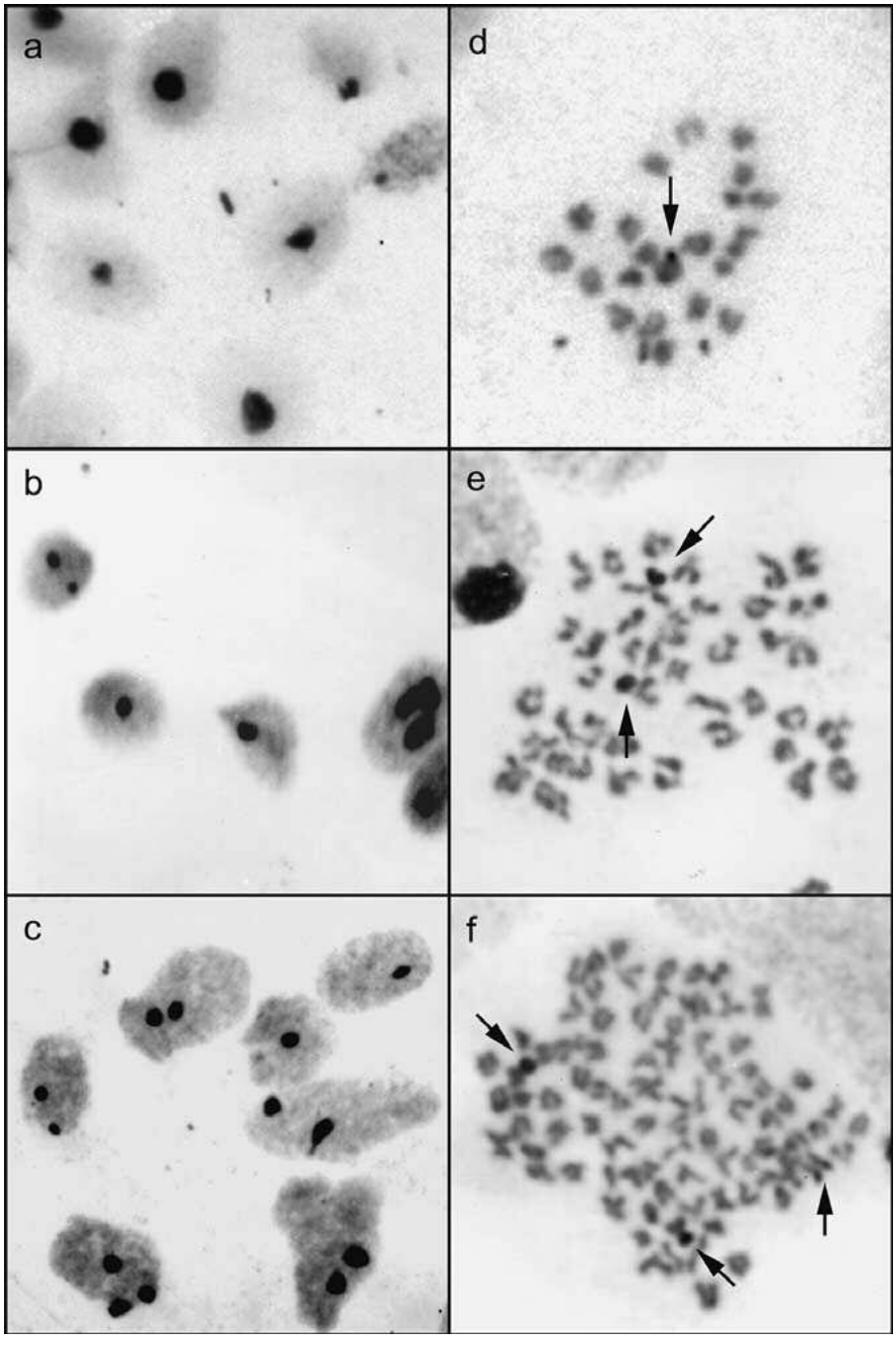

Figure 1. Interphase nuclei (a, b, c) and metaphase plates (d, e, f) obtained following the procedure of Kligerman and Bloom (1977) on turbot embryos (n) or larvae (2n, 3n) and treated with silver staining (Howell and Black, 1980). Diploid gynogenetics (b, d) and haploid (a, d) and triploid (c, f) controls corresponding to one of the families analyzed are presented. Arrows indicate the locations of NORs.

0.90, with mean figures of 0.803 and 0.769, respectively. The potential for paternity testing was assessed by computing the 2 exclusion probabilities for each locus and over all loci. When only the genotypes of the offspring were known, the combined probability over loci to exclude a nontrue candidate parent was 99.897%. When the genotype of one parent was known, the probability of exclusion reached 99.999%.

With this level of exclusion, virtually any paternal contribution could be detected in the gynogenetic offspring once the genotype of the mother was known. Two diagnostic microsatellite loci were selected in each cross to check the exclusive maternal contribution to gynogenetic offspring. PCR amplifications of these 2 loci in about 20 gynogenetic offspring in each cross are summarized in Table 3, and representative gels in a subsample of these crosses are shown in Figure 2. Offspring genotypes exclusively showed maternal alleles with no paternal genetic contribution, suggesting a 100% gynogenetic performance. When the genotype of the mother was heterozygous, the 2

Table 1. Mean, Standard Error, and Range of the Average Number of Nucleoli per Nucleus in 20 Specimens Analyzed in Each Group (n, 2n, and 3n) in the Two Experiments for Obtaining Gynogenetic Offspring

	Experiment 1		Experiment 2	
Group	$\bar{X} \pm$ SE	Range	$\bar{X} \pm$ SE	Range
n	1.031 ± 0.018	1.00–1.20	1.333 ± 0.010	1.21–1.41
2n	1.442 ± 0.017	1.36–1.58	1.558 ± 0.022	1.37–1.73
3n	2.008 ± 0.026	1.80–2.32	1.952 ± 0.030	1.75–2.29

Table 2. Estimates of Genetic Diversity and Probabilities of Exclusion in an Atlantic (NW Spain) Population of Turbot (*Scophthalmus maximus*) for the 11 Microsatellite Loci[a]

Locus	NA	H_e	PIC	Excl(1)	Excl(2)
Smax-01	10	0.764	0.720	0.369	0.546
Smax-02	18	0.824	0.805	0.507	0.679
Smax-03	7	0.639	0.577	0.225	0.386
Smax-04b	12	0.769	0.739	0.399	0.583
Sma3-8INRA	13	0.866	0.843	0.563	0.723
Sma3-12INRA	11	0.819	0.786	0.460	0.634
Sma4-14INRA	10	0.797	0.761	0.422	0.600
Sma5-111INRA	13	0.898	0.877	0.628	0.773
Sma1-125INRA	9	0.755	0.710	0.351	0.530
Sma3-129INRA	16	0.889	0.868	0.613	0.761
Sma1-152INRA	8	0.812	0.776	0.442	0.618
Mean/total	11.545	0.803	0.769	0.998971	0.999985
SE	1.003	0.022	0.026		

[a]NA, allele number; H_e, expected heterozigosity; PIC, polymorphic information content; Excl(1), exclusion probability for the first parent; Excl(2), for the second parent. The mean over loci with its standard error for genetic diversity estimates, and the exclusion probabilities for all loci are also included. Calculations were performed with the computer program 2.0 (Marshall et al., 1998). PCR amplification for all loci is described in Bouza et al. (2002).

expected homozygotes and a variable proportion of heterozygous offspring were found, evidencing recombination events between the locus and the centromere.

Discussion

Ploidy determination is the usual approach to check the success of gynogenesis experiments. When possible, karyologic observation constitutes a direct and accurate method to establish the ploidy level at any step of the process.

Table 3. Genotypes of the Father, Mother, and Offspring for the Two Diagnostic Loci Selected at Each Cross for Checking Exclusive Maternal Transmission in Gynogenetic Offspring

Experiment	Locus	Father	Mother	Off spring[a]
1	*Smax-02*	95/95	109/133	109/109 (6)
				109/133 (7)
				133/133 (6)
	Sma1-125INRA	132/136	120/120	120/120 (19)
2	*Sma3-8INRA*	194/202	186/196	186/186 (7)
				186/196 (5)
				196/196 (8)
	Sma3-129INRA	179/185	169/169	169/196 (20)

[a]In parentheses are given are numbers of individuals observed for each genotype.

However, obtaining metaphase plates of sufficient quality for karyotype analysis has been a challenge in many fish species. The development of reliable cell culture techniques has improved cytogenetic studies in fish, although these techniques are far from those applied routinely in higher vertebrates (Hartley and Horne, 1985; Gold et al., 1990; Martínez et al., 1993). The nonviability of the gynogenetic haploids and the need for quick determination of the ploidy level when setting up a method to induce gynogenesis in fish limit the use of these cell culture techniques. Classical methods based on obtaining metaphases directly from solid tissues (Kligerman and Bloom, 1977) are available in embryos or larvae. The quality of the metaphases obtained and the variable performance in individual fish limit the routine use of this technique for checking ploidy level. In addition, cell culture of kidney or spleen from adults, probably the best tissues for obtaining good metaphase plates in fish (Martínez et al., 1993), requires sacrifice, which prevents its use for gynogenetic testing in adults.

Silver staining for localizing NORs and nucleoli has provided an inexpensive and straightforward method to overcome the limitations of chromosome counting (Phillips et al., 1989). When NOR regions are located in a single chromosome pair, a maximum of 1, 2, and 3 nucleoli are expected in haploid, diploid, and triploid cells, respectively. Under these considerations, this technique has been successfully applied in some fish species (Felip et al., 2001), including the turbot (Piferrer et al., 2000). The problem arises when multichromosomal location or polymorphism of NORs exists in the species under study. Piferrer et al.

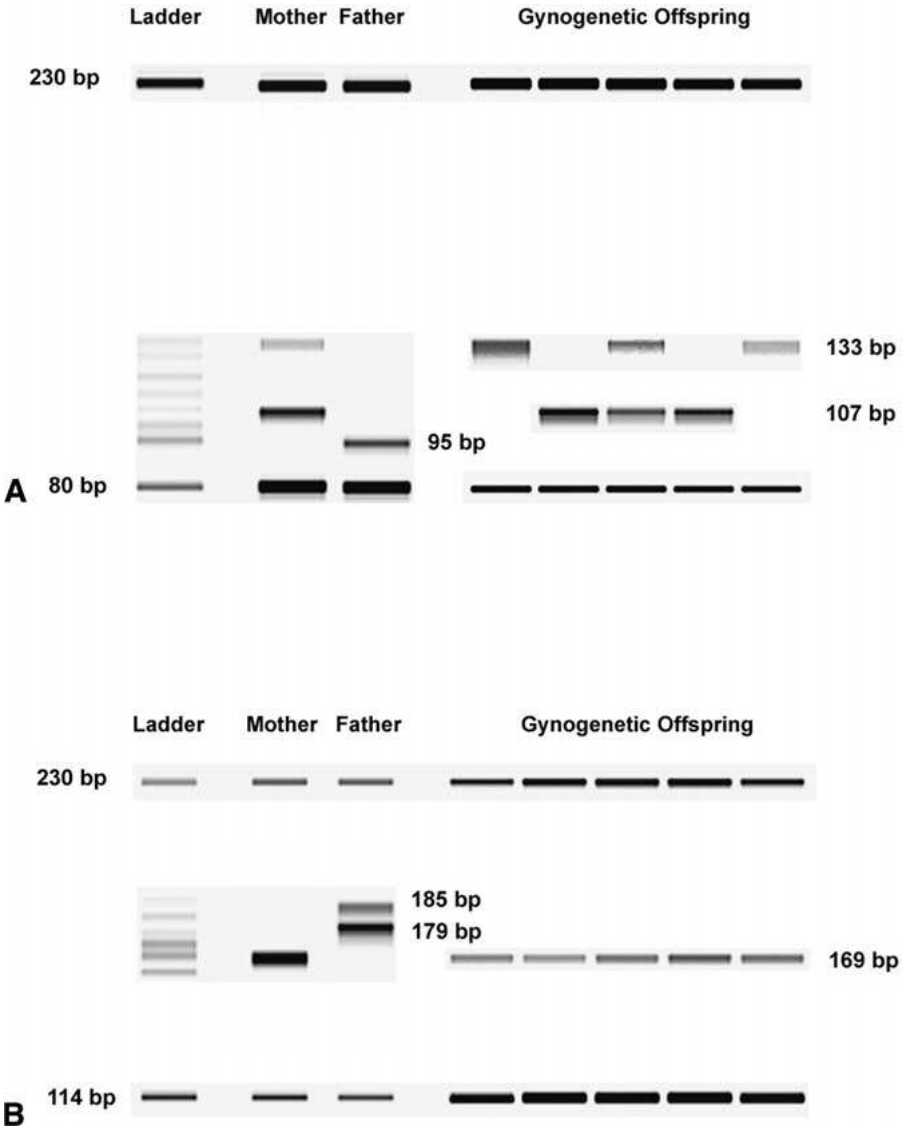

Figure 2. Demonstration of exclusive maternal contribution to gynogenetic offspring using the diagnostic microsatellite loci *Smax-02* (**A**) and *Sma3-129INRA* (**B**) in each of the 2 families analyzed, respectively. The electrophoretic patterns presented show, in lane 1, the external standard size marker including an allelic ladder. Additionally, each lane contains 2 internal standard size markers (A, 80–230 bp, B, 114–230 bp) for genotyping accuracy. Lanes 2 to 8 contain correlatively the electrophoretic patterns of the 2 parents (A, 107/133 female and 95/95 male; B, 169/169 female and 179/185 male) and a sample of 5 gynogenetic offsprings (A, 107/107, 133/133, 107/133; B, all 169/169).

(2000) have shown that distributions of the number of nucleoli per cell in triploids and diploids in turbot overlapped, leading to about 3% error in the assessment of ploidy level. In this species, besides the main NOR-bearing submetacentric chromosome pair (Bouza et al., 1994), different acrocentric chromosomes have occasionally showed silver staining at their telomeres (Bouza, 1994; Pardo et al., 1998). This can constitute an important disturbance in the evaluation of the ploidy level in turbot.

In the first cross performed in this study, no overlapping was observed in the ranges of nucleoli per nucleus between any of the 3 distributions (haploids, diploids, and triploids), each individual being unambiguously assigned to its ploidy level. However, in the second cross, the mean numbers of nucleoli per nucleus in haploids and diploids were higher than in the first cross, with cells with 2 and 3 nucleoli in haploids and diploids, respectively, and both distributions evidenced overlapping. The misclassification of individuals according to their ploidy is small, as pointed out by Piferrer et al. (2000); however, NOR analysis does not guarantee an errors-proof individual identification, which is essential for the establishment of a gynogenetic broodstock in turbot. NOR analysis is still valid for estimating the success of a particular ploidy manipulation in a sample (percentage of individuals with the expected ploidy), but caution must be taken with the results obtained.

Exclusive maternal genetic transmission to the offspring during the induction of gynogenesis, although highly correlated with ploidy estimation, is determined by a different approach. None of the methods described above can unambiguously confirm the gynogenetic nature of the offspring, especially when the efficiency of the techniques

involved in gynogenesis is far below 100%. Specific morphologic characters with simple inheritance models (Nagy et al., 1978; Don and Avtalion, 1988) and biochemical markers like allozyme loci (Thorgaard, 1983; Sugama et al., 1992; Yousefian et al., 1996) have been the only alternatives to verify maternal inheritance in fish until recently. However, the low genetic variability exhibited by these markers and the necessity of sacrificing the individuals analyzed limit their use. Polymorphic DNA markers have arisen in the last decade as the best alternative to address questions of relevance for managing fish populations (O'Reilly and Wright, 1995; Ferguson and Danzmann, 1998). Their high polymorphism and the simplicity of DNA extraction account for their growing use for genetic identification, parentage, or pedigree analysis in the last years. Determination of the true gynogenetic nature of offspring is essentially a parentage analysis and, hence, affordable with the use of DNA genetic markers. In this sense, Felip et al. (2000) and Peruzzi and Chatain (2000) have demonstrated exclusive maternal inheritance in gynogenetic diploid sea bass by using AFLPs and microsatellite analysis, respectively.

In the present work we used a set of 11 polymorphic microsatellite loci to verify maternal inheritance in gynogenesis experiments in turbot, overcoming the limitations of analysis by the number of nucleoli detected in this species. The microsatellite loci used demonstrated high variability in several populations studied previously (Coughlan et al., 1996; Estoup et al., 1998; Bouza et al., 2002). This genetic variability permits a high level of confidence in paternity testing, as indicated by the 2 exclusion probabilities obtained: 99.897% when only the offspring genotypes are known and 99.999% when the genotype of one parent is also known. The last figure implies that if the genotype of the mother and her offspring is known (the normal situation in induced gynogenesis), then virtually any paternal genomic contribution can be detected. In practice, however, it is sufficient to analyze only 2 carefully selected diagnostic loci in the offspring to confirm exclusive maternal contribution. Selection of 2 loci is recommended, taking into consideration the mutation rate at these loci, and the possible existence of paternal residual transmission in some offspring analyzed (Thorgaard et al., 1985).

The results of this work have demonstrated the potential of highly variable microsatellite loci to monitor uniparental inheritance when inducing gynogenesis in fish, avoiding the problems related to the existence of polymorphism for NOR regions, as in the case of turbot.

Acknowledgements

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Artículo 7

Growth and gonadal development of gynogenetic diploid turbot (*Scophthalmus maximus*)

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GROWTH AND GONADAL DEVELOPMENT OF GYNOGENETIC DIPLOID TURBOT (*Scophthalmus maximus*)

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Abstract

The aim of this study was to determine the survival, growth, gonadal development, and sex ratios of gynogenetic diploid turbot between 9 and 36 months of age. Gynogenesis was induced by activation of freshly collected eggs with diluted (1:10 with Ringer) and UV-irradiated (30,000 erg.mm^{-2}) sperm, followed by a cold shock at –1°C to 0ºC for 25 min, starting at 6.5 min after fertilization. The exclusive maternal inheritance of the resulting gynogenetic diploids was confirmed in all individuals used in the experiment by microsatellite markers. Mean length and weight throughout the experiment was higher in the controls than in the gynogenetics. The gonadal development in gynogenetic males proceeded normally, and in gynogenetic females it was delayed during the first sexual maturation but was normal during the second one. The sex ratio was 1 male (M):1 female (F) in the controls, as expected, but 1 M:3 F in the gynogenetics. However, in a second, independent experiment, carried out with fish originating from different broodstock, the sex ratio was 1 M:1 F in the controls and 0 M:1 F (i.e., 100% females) in the gynogenetics. Together, these results suggest that the main sex-determining genetic component in turbot fits well to female homogamety, in accordance with data from other flatfishes. These results indicate that it will be possible to obtain near or all-female turbot stocks through induced gynogenesis or by crosses involving hormonally sex-reversed fish.

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1. Introduction

Gynogenesis induction (production of offspring with exclusive maternal inheritance) is used in fish genetic improvement programmes as the first step towards the production of monosex female stocks, to achieve highly inbred lines, or as a tool to draw genetic maps (Ihssen et al., 1990; Mair, 1993; Felip et al., 1999; Danzmann and Garbi, 2001).

Gynogenetic fish have already been artificially produced in several freshwater and marine species for basic and applied research (Thogaard, 1983; Solar et al., 1991; Felip et al., 2001). Currently, gynogenesis is used in the aquaculture of rainbow trout (*Salmo trutta* L.) in France, common carp (*Cyprinus carpio* L.) in China and Japan, and Japanese flounder hirame (*Paralychthys olivaceus*) Temminck & Schlegein in Japan (Hulata, 2001).

Preference for male or female monosex stocks depends on the species growth performance in culture. In many fish species of commercial interest, females exhibit higher growth rates. The artificial production of monosex populations depends on the sex determination mechanism. Only species with a sex determination system XX (females) / XY (males) can produce exclusively female gynogenetic offspring (Devlin and Nagahama, 2002). Nevertheless, Felip *et al*. (2001) have listed the presence of males in gynogenetic stocks from different species apparently having an XX/XY sex determination mechanism. The existence of other minor sex determining genes as well as the effects of environmental factors such as temperature, could explain the presence of males among gynogenetics in these cases.

Although the induction of gynogenesis has been reported for many fish species, most of the experiments studied the effects at the larval or juvenile stages. This is the reason why few data are available on the actual growth and gonadal development of gynogenetic diploid fish, specially regarding marine species (Felip *et al*., 2002).

Turbot (*Scophthalmus maximus* L.) shows an important differential growth in favour of females (Imsland *et al*., 1997). Because of this, the production of all female stocks represents an interesting option for the aquaculture of turbot. Methods for the induction of gynogenesis in turbot have recently been described by Piferrer *et al*. (2004), including the viability and survival of gynogenetic progenies until an age of six months.

In this work, survival, growth and gonadal development of 33 gynogenetic diploid and 33 control turbots from the same family were studied from 6 to 36 months of age (adults), encompassing two full maturation cycles. We also report the sex ratio of gynogenetic diploid turbot obtained in two independent experiments. Results support the idea of using gynogenetic diploids to obtain all-female stocks for turbot aquaculture.

2. Materials and methods

2.1. *Animals used*

The control diploid turbot ("controls") and the meiogynogenetic diploid turbot ("gynogenetics") used in the present study have been described in detail elsewhere, covering the period comprised from fertilization to six months of age (Piferrer et al., 2004). Briefly, gynogenesis was induced by the use of sperm (diluted 1:10 with Ringer 200) that had been irradiated (30,000

erg. mm^{-2}) with UV-light to fertilize freshly collected eggs. Diploidy was restored by applying an optimized cold shock of –1ºC to 0ºC for 25 min, starting 6.5 min after fertilization at 13-14°C to retain the second polar body (Piferrer et al., 2003). In this way, we obtained a large amount of gynogenetic turbot. We used these fish primarily to document possible abnormalities during embryogenesis, to establish the Hertwig effect and to study their early survival and growth until they were weaned into artificial food. This is reported elsewhere (Piferrer et al., 2004)

The remaining fish was used to study their actual gynogenetic origin and also used in the present study. The actual gynogenetic origin of the fish obtained by UV-irradiation of the sperm followed by cold shock of the eggs was assessed in a sample of two-day-old larvae by the use of a set of eleven microsatellite markers developed specifically for this purpose and reported elsewhere (Castro et al., 2003). After genotyping 11 microsatellite loci in the parents involved in each family, two diagnostic microsatellite loci (not shared by male and female) were selected for checking exclusive maternal inherence in offspring.

The non-sacrificed fish available (n=33 confirmed gynogenetics plus an equivalent number of diploids) constituted the first one of the two families used for the present study. The gynogenetic nature was re-confirmed in five-month-old fish. Thus, all "gynogenetics" described in the present study were in fact 100% gynogenetic diploids.

At nine months of age, the study reported here started. After being vaccinated against *Vibrio sp* with Gava-3 (Laboratorios HIPRA, Barcelona, Spain) as is usual for this species, 33 controls and 33 gynogenetics with the same initial mean weight and length were individually tagged using passive internal (PIT) tags (Indexel C 413420), placed in separate tanks and cultured according to the usual procedures for turbot (Iglesias et al., 1987; Sánchez, 1990; Sánchez et al., 1990). Both groups of fish were cultured separately in order to avoid any possible dominance effect (competence for food) of controls over gynogenetics or vice versa. Tanks had an open circuit of seawater, and fish were fed using automatic feeders with commercial dry feed seven days a week, during the first year, and three days a week during the second and third years.

Survival was determined during each sampling and at the end of the experiment, excluding those individuals that were sacrificed for the GSI and HSI determinations.

2.2. Growth and body indices

All fish were periodically sampled from 9 to 36 months of age to monitor changes in length and weight. Total length (cm) and average weight (g) were calculated during each sample and differences were statistically compared between both ploidies. The condition factor (K) was calculated as weight (W) divided by length (L) cubed (W/L^3). Specific growth rates for weight (SGRw) and length (SGR_L) were calculated for each period between samplings as ($SGR_W = 100$ (Ln Wt - Ln Wi) / t and $SGR_L = 100$ (Ln Lf - Ln Li) / t, where Wt and Lt are final weight and length, and Wi and Li are initial weight and length for the given period of t days. At 24 and 36 months of age, the gonadosomatic (GSI) and hepatosomatic (HSI) indices were determined in 10 gynogenetics (4 males and 6 females) and 10 controls (4 males and 6 females) as the weight of gonads or liver, respectively, divided by the body weight.

2.3. Gonadal development

From an age of 18 months onwards, gonadal development was studied by observing the swollen abdomen in females and by checking sperm production in males. This allowed determining the sex of most individuals, but this could only be confirmed once they were sacrificed at the end of the experiment. The percentage of spermiating males and ovulated females was determined at 24 and 36 months of age. gametes were fertile, eggs were obtained by gentle abdominal massage, and the fertilization ability of sperm obtained from spermiating males was determined

To determine if gynogenetic turbot gonads were functional enough to allow them to produce gametes, i.e., simply capable of producing eggs and sperm and if this on those eggs. Also, at an age of 24 and 36 months, coinciding with the first and second sexual spawning season, respectively, 10 individuals per group were sacrificed (the same individuals used for the GSI and HSI determination), and samples from their gonads were fixed in glutaraldehyde in order to study gonadal morphology by routine histological procedures.

2.4. Sex ratios

The sex ratio was calculated at the end of the experiment with fish aged 36 months, using the cumulative data obtained from the fish sacrificed at 24 and 36 months, plus data from the adult fish (non sacrificed) sexed with absolute certainty while in the process of maturing.

The sex ratio was also determined in a second group of gynogenetic turbot and their corresponding controls. This group was originated from a different set of parents. Gynogenesis was induced exactly in the same manner as described above and the actual gynogenetic nature of the fish obtained was also verified as above, i.e., by the use of microsatellite markers, first in a sample of 20 two-day-old larvae and then in 80 six-month-old fish. In this case, sex ratios were determined histologically in n = 80 six-month-old fish, once the gonads had sexually differentiated and the sex could be unambiguously determined.

2.5. Statistical analysis

Growth in length and weight at a given age between controls and gynogenetics was compared by the Student's t-test, taking into account the normality of the data distribution and the homogeneity of variances checked by using Levené's test (Sokal and Rohlf, 1986). The Kruskall Wallis nonparametric test of averages was used to compare the body indices (IGS and HIS) between gynogenetics and controls in both sexes. The null hypothesis deviation of the 1:1 expected sex ratio was tested with the Chi-square test. Differences were considered significant when $P<0.05$. Data were expressed as mean ± SEM.

3. Results

3.1. *Survival*

From 9 to 36 months of age, the survival of controls was 96.7% and of gynogenetics 87.9%.

3.2. *Growth*

Mean length and weight throughout the experiment was higher in the control group than in the gynogenetics both for females (Fig. 1A and C) and males (Fig. 1B and D), except in weight for gynogenetics at 36 months of age. The difference in length was significant ($P<0.05$) in the period from 15 to 30 months (both included) of age in females, and from 12 to 24 months of age in males. The difference in weight was significant ($P<0.05$) from 21 to 24 months of age in females and from 15 to 21 months of age in males.

The condition factor throughout the experiment both for females (Fig. 1E) and males (Fig. 1F), was higher in gynogenetics than in the controls. The difference in condition factor was significant in females from 15 to 18 and at 24 months of age ($P<0.05$). In males, the difference was significant at 15 and from 24 to 36 months of age ($P<0.05$).

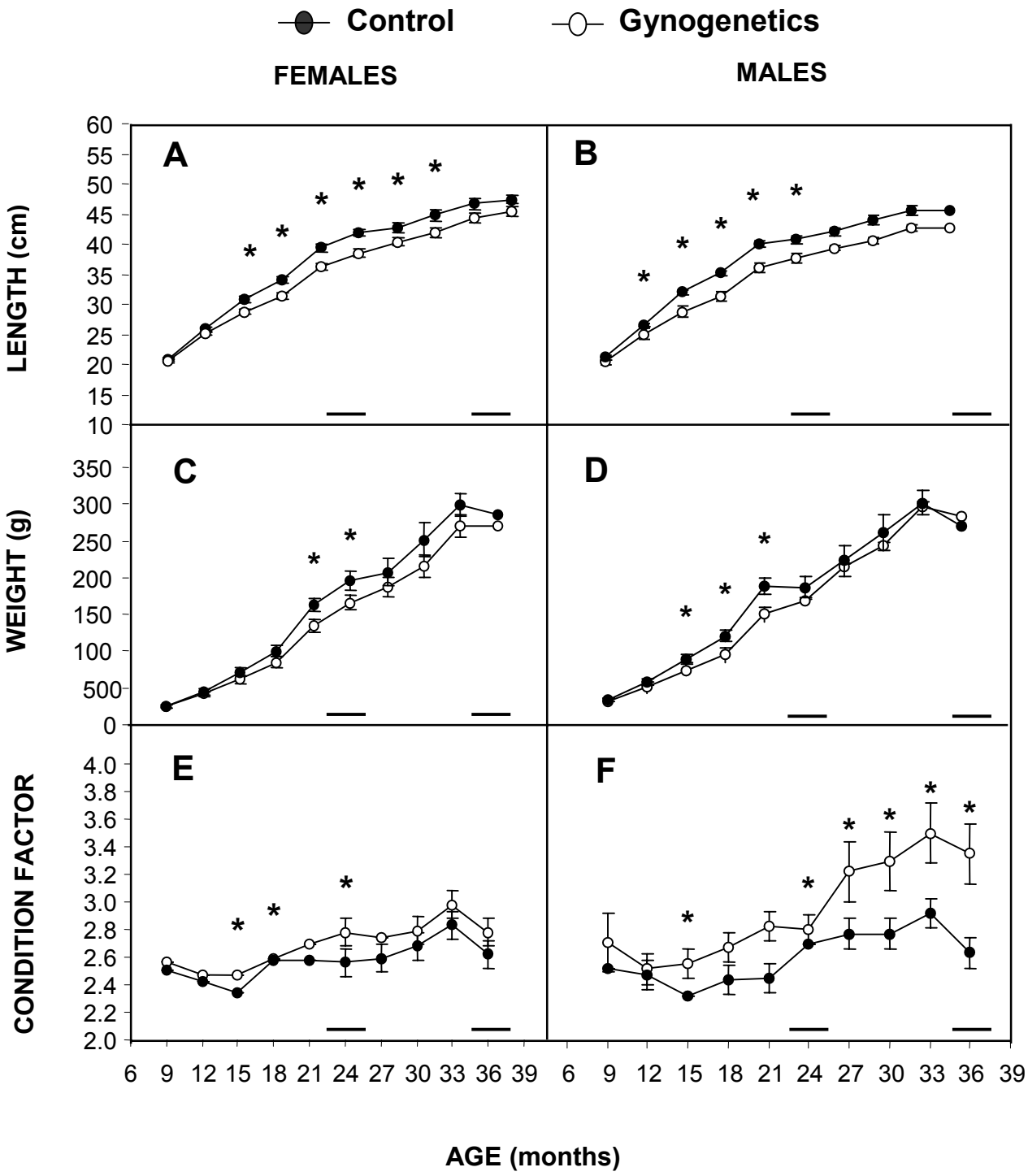

Fig. 1. Growth in length (A,B), weight (C,D), and condition factor (E,F) of female and male diploid and gynogenetic diploid turbot between 9 to 36 months of age. Asterisks indicate significant ($P<0.05$) differences between the groups. Bars indicate the spawning season. Data as mean ± SEM.

The specific growth rate in both groups decreased for both length (Fig. 2A and B) and weight (Fig. 2C and D) throughout the experiment. The lowest values were recorded during the months coincident with -or immediately subsequent to- the spawning seasons. The decrease in the instantaneous growth rate in weight was more marked in the controls than in the gynogenetics.

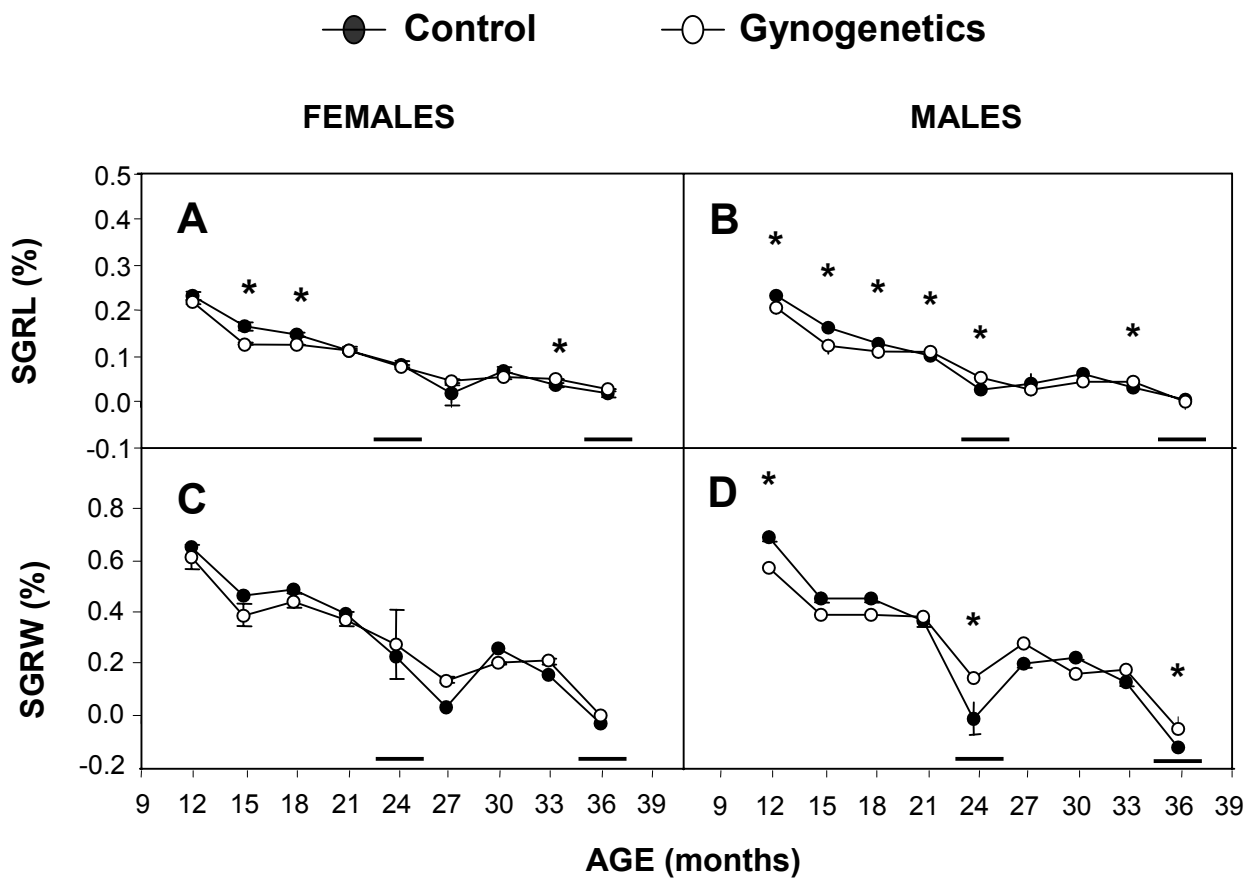

Fig. 2. Specific growth rate in length (SGRL) (A,B) and weight (SGRW) (C,D) of female and male diploid and gynogenetic diploid turbot between 9 to 36 months of age. Asterisks indicate significant ($P<0.05$) differences between the groups. Bars indicate the spawning season. Data as mean ± SEM.

3.3. Gonadosomatic and hepatosomatic indices

No significant statistical differences were observed between controls and gynogenetics in GSI ($P>0.05$) (Fig. 3A) and HSI ($P>0.05$) (Fig. 3B), regardless of sex at 24 or 36 months of age.

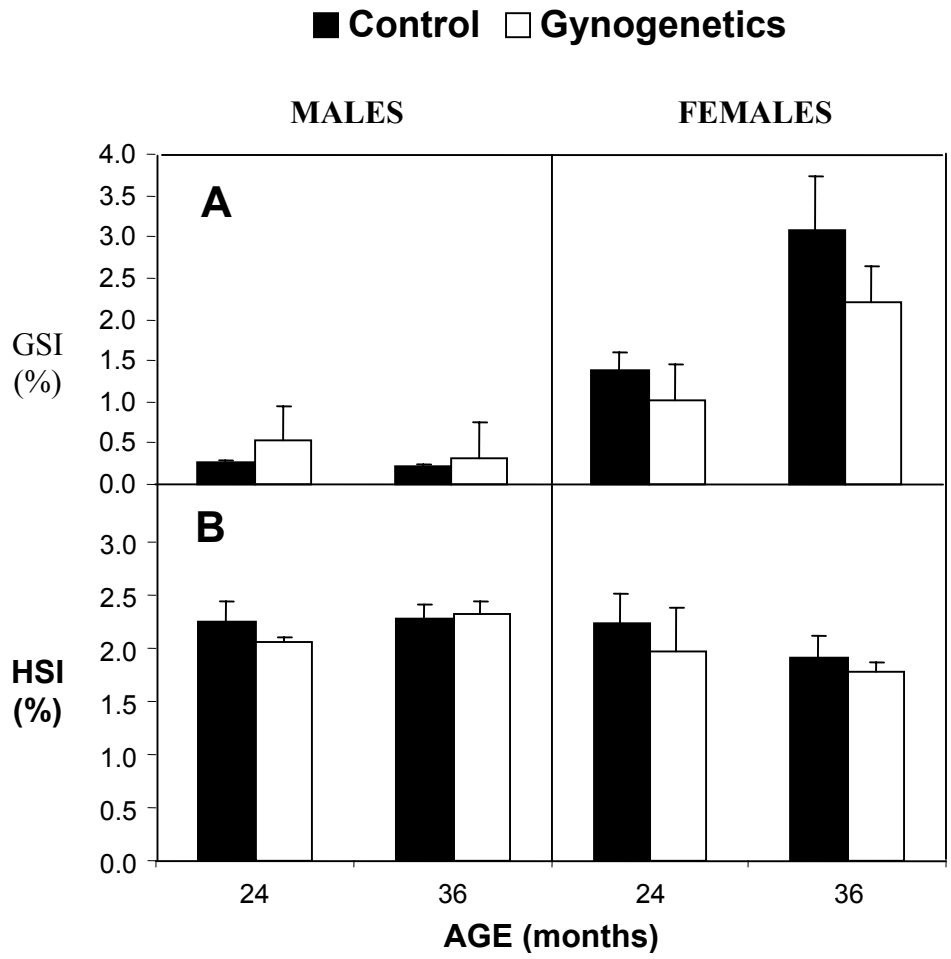

Fig. 3. Gonadosomatic (GSI) (A) and hepatosomatic (HIS) (B) indices of male and female diploid and gynogenetic turbot at 24 and 36 months of age. Data as mean + SEM.

3.4. Gonadal development

Gonadal development in gynogenetics both in males and females was basically normal from a histological point of view, but with certain delay in its timing, particularly in females.

At 24 months of age, external signs of sexual maturity (i.e., the emission of milt upon gentle abdominal massage) were observed in 100% (n=17) of the control males (Fig. 4). On the other hand, only 42.8% (n=3) of the gynogenetic males ($P<0.05$) produced sperm at this age, and in less amount. In the control females, 53% (n=15) of them had a swollen abdomen and 13% (n=2) released eggs. In the gynogenetic females, 16.7% had swollen abdomen but none of them released eggs ($P<0.05$) (Fig. 4).

Presence of spermatids and spermatozoa at 24 months was observed by histological analysis in the testes of control males (Fig. 5A) and in the gynogenetic diploids (Fig. 5B). However, as judged by representative photomicrographs, in the gynogenetics the amount of spermatozoa was less than in the controls. In contrast with the situation in males, clear differences were observed in the gonadal development of females from both groups. Thus, while it was easy to identify

many mature oocytes in the ovaries of the control females (Fig. 5C), in the gynogenetic diploid females only oocytes in the perinucleolar stage could be identified (Fig. 5D).

At 36 months of age, 100% (n=9) of the males and 67% (n=12) of the females in the controls attained sexual maturity, producing sperm and eggs, respectively. In the gynogenetics, 100% (n=2) males also produced sperm ($P>0.05$) but only 17.6% (n=17) of females produced eggs ($P<0.05$). (Fig. 4)

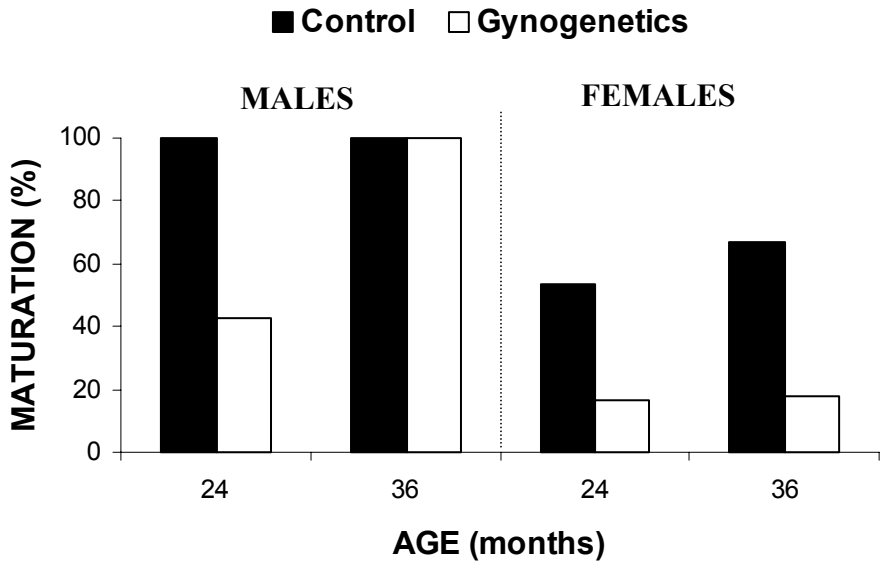

Fig. 4. Percent maturation of control and gynogenetic turbot according to sex during their first(24 months of age) or second spawning season (36 months of age). Asterisks indicate significant ($P<0.05$) differences.

Presence of spermatids and spermatozoids at an age of 36 months was observed by histological analysis in all samples from control and gynogenetic males (Fig. 6A and B). In the females (Fig. 6C and D), presence of mature oocytes was observed in all samples taken from the controls, but only in some of the gynogenetic females, while in the remaining gynogenetic females only oocytes in the perinucleolar stage were observed.

In order to check if gynogenetics turbot gametes were functional to allow fertilization, eggs obtained from one gynogenetic female were fertilized with sperm obtained from one gynogenetic male following standard procedures. The observed fertility (23.2%) and embryonic development until the larval stage (4.9%) were normal for gynogenetic turbot (Piferrer et al., 2004), showing the ability of these fish to produce viable gametes in a similar way, albeit in less quantity, than regular diploids.

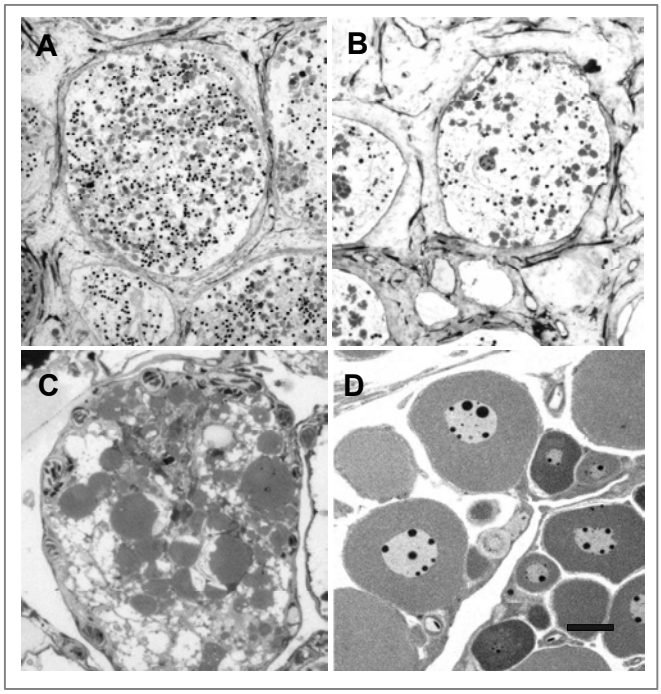

Fig. 5. Histological sections of gonads from diploid and gynogenetic diploid turbot of 24 months of age. A, diploid testes; B, gynogenetic diploid testes; C, diploid ovary; D, gynogenetic diploid ovary. Bar= 30 μm (for A and B); 70 μm (for C and D).

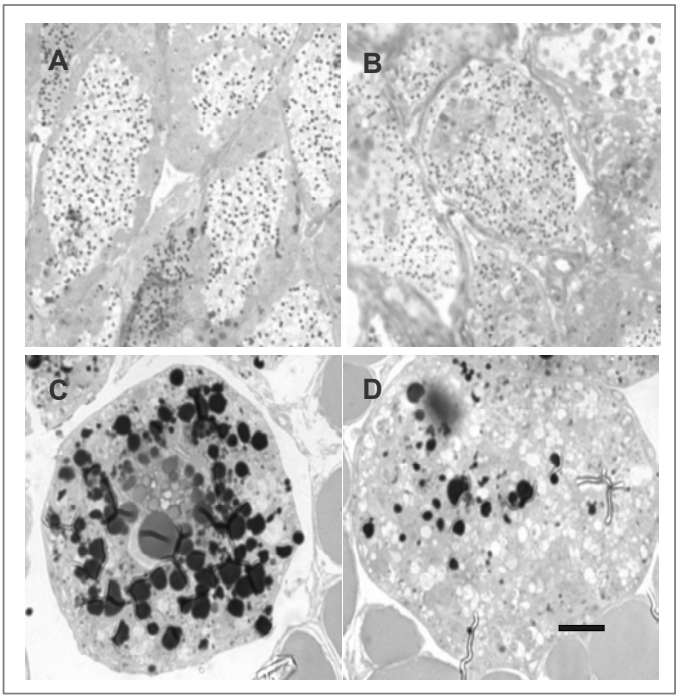

Fig. 6. Histological sections of gonads from diploid and gynogenetic diploid turbot of 36 months of age. A, diploid testes; B, gynogenetic diploid testes; C, diploid ovary; D, gynogenetic diploid ovary. Bar= 30 μm (for A and B); 80 μm (for C and D).

3.5. Sex ratios.

The sex ratio was 1 male (M):1 female (F) in the controls (n=33), as expected, and not differing from 1 M:1F, but 1 M:3 F in the gynogenetics (n=33) ($P<0.001$). However, in the second, independent experiment, the sex ratio was 1 M:1 F in the controls (n=80) and 0 M:1 F (i.e., 100% females) in the gynogenetics (n=80) (Fig. 7).

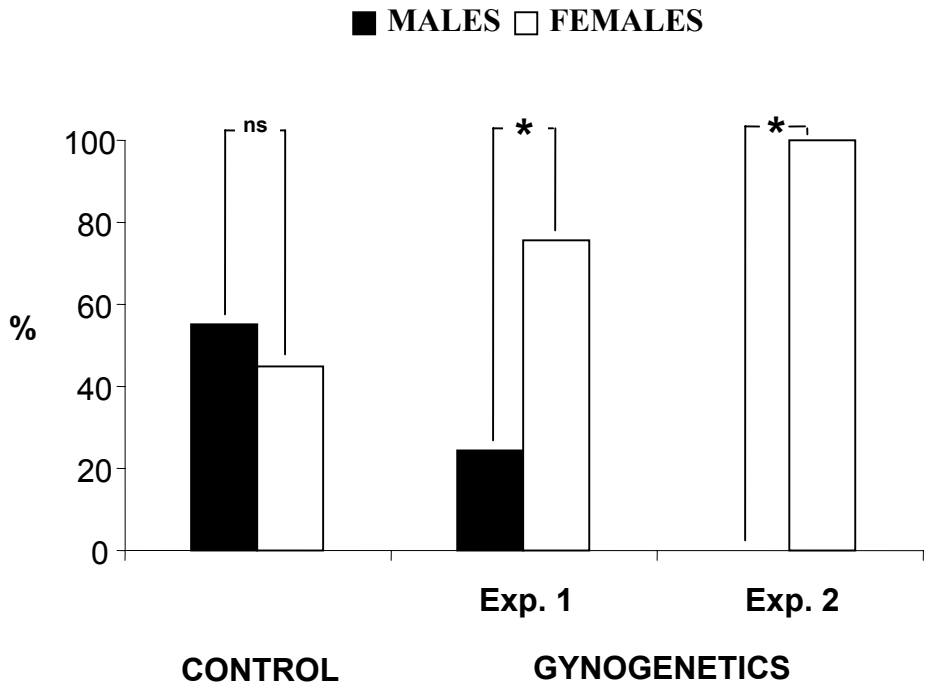

Fig. 7. Percent maturation of control and gynogenetic turbot according to sex during their first (24 months of age) or second spawning season (36 months of age). Asterisks indicate significant ($P<0.05$) differences.

4. Discussion

The objective of this research was to study the viability, growth, sex ratios and gonadal development until maturity of gynogenetic diploid turbot, with the aim to 1) determine the major biological consequences of induced gynogenesis for its application in culture, and 2) obtain relevant information to elucidate the sex determination mechanism for this species.

In all fish species studied so far, lower hatching and survival rates of gynogenetic diploids are evident during the first year of life, specially in the larval stage and the transition to pelleted food (Cherfas, 1981). The lower survival of gynogenetics is probably due to the expression of deleterious recessive alleles as a consequence of the higher inbreeding typical of gynogenetic fish (Leary et al., 1985; Don and Avtalion, 1988; Ihssen et al., 1990). Gynogenetic diploid turbot also showed lower hatching and early survival rates (Vázquez et al., 2002; Piferrer et al., 2004). In this regard, gamete manipulations during the induction process exert a very negative effect on their viability, specially in marine species (Felip et al., 2001). In turbot, the oligospermic nature of males (males having a very small sperm production volume) (Suquet el al., 1994) constitutes an additional problem to the manipulation of gametes.

Survival of gynogenetic individuals varies considerably, with important differences even between females from the same group (Cherfas, 1975). For turbot aged six to 36 months, survival of gynogenetic diploids was about 10% lower than that of the controls. In addition, gynogenetic turbot was similar to the control diploid in growth performance, and only significant weight differences were observed during the months leading to the first sexual maturation. This indicates that, despite the higher incidence of inbreeding, they are still able to exhibit nearly normal growth and development rates. At the present time, there are not enough studies to allow concluding whether the effects of gynogenesis on growth have a similar pattern regardless of species. Thus, while for carp (Cherfas, 1975) and seabass (Felip *et al*., 2002) no significant differences were observed between the controls and the gynogenetics, in the case of other species, such as the Atlantic salmon (Johnstone and Stet, 1995), gynogenetic diploid individuals grew less than the control diploids.

The greater value of the condition factor of gynogenetic turbot with respect to the controls probably reflects the fact that gynogenesis affects growth in a different way whether weight or length is considered. Gynogenetic fish show a slightly lower growth rate due to their condition, which is reflected in a slightly minor length. Nevertheless, growth in weight was not so affected as it could be expected, perhaps because is relatively more susceptible to environmental factors, thus reducing the relative influence of the genetic contribution. This would explain the higher condition factor of gynogenetic turbot relative to that of the diploids. Another possibility, however, is that the higher condition factor in the gynogenetics is due to their less advanced stage in gonadal maturation (vide infra).

Body indices (IGS and IHS) measured at an age of 24 and 36 months did not show significant differences between control and gynogenetic diploids, indicating no major apparent effects of gynogenesis on hepatic function, also reflecting the ability of gynogenetic females to undergo vitellogenesis.

Alterations in the gonadal development have been observed in induced gynogenetic diploids from different species such as carp (*Cyprinus carpio* L) (Nagy *et al*., 1978) and coho salmon (*Oncorhynchus kisutch W.*) (Piferrer *et al*., 1994). Not all turbot achieve sexual maturity at 24 months. Gynogenesis increased the amount of fish with delayed gonadal maturation. This suggests an effect of the lower heterozygosis of gynogenetics on the normal development of the male and female gonad.

Sex ratios of gynogenetic diploids can provide relevant information on the sex determining mechanism of a particular species. For example, induced gynogenesis resulted in 100% females in the silver barb, (*Puntius goniotus B.*) (Pongthana et al., 1995), indicating female homogamety, but in the common sole (*Solea solea L.*) induction of gynogenesis resulted in the appearance of both sexes (Howell et al., 1995).

In the turbot, the sex ratio in the controls was 1M:1F, in accordance with what had previously been described for this species by Imsland et al. (1997). The sex ratio (3F:1M) observed in the first gynogenetic batch is not very consistent with a XX/XY or ZZ/ZW sex determination mechanism, although the higher proportion of females suggests an XX/XY mechanism rather than an ZZ/ZW one. Other factors like minor autosomal genes would be necessary to explain the presence of 25% of males in the gynogenetics. Nevertheless, the presence of males in the gynogenetics of many species that are known to have female homogamety has been previously reported (Felip et al., 2001). Additional data from triploid turbot (3F:1M; Cal et al., 2004), does not adjust perfectly to an XX/XY classical determining mechanism, and suggest some kind of additive genetic effect on sex determination. The greater the number of female genomes, the higher the proportion of females obtained in triploids.

Support for female homogamety in our study was provided by the assessment of sex ratios in a different batch of fish where gynogenetics were all-female. Thus, with the results of family two, which gave 100% females we are confident in assuming that this is good proof of female homogamety in the turbot. This is so because after the induction of gynogenesis in fish one can obtain 100% females only if the sex determination system is of the type XX female XY male. To the best of our knowledge there is not a single report in the literature that 100% females re obtained after gynogenesis induction in fish if that is not the sex determination system.

Thus, viewed together, these results suggest that most likely turbot exhibit female homogamety, in accordance with what has been reported in other flatfishes (*Limanda yokohamae* (Aida, S. & Arai K. 1998), *Paralichthys olivaceus* (Tabata, K. 1991), *Microchirus ocellatus L.* and *Bothus podas D. (*Vitturi et al., 1993a and 1993b, respectively). However, other minor autosomal factors, additive genetic component and/or environmental factors are probably involved in sex determination in turbot, as observed in several teleosts with female homogamety, where gynogenetic progenies may not always be 100% females.

These results as a whole show the feasibility to produce gynogenetic diploid turbot to commercial size. Although their viability and growth could make them in principle suitable for production, their high number of females suggests that the best option would be to use gynogenetics as an excellent starting point to obtain all-female stocks. We agree that in order to study whether gynogenetic turbot are equally fertile than normal diploids by performing only one crossing is certainly not enough. But that was not our goal. Our objective was to determine if gynogenetic turbot gonads were functional enough to allow them to produce gametes, i.e., simply capable of producing eggs and sperm. This is the necessary requisite for their potential use as broodstock in a production scheme as gamete donors for monosex production, since gynogenetic fish cannot be directly used as production fish due to their lower viability. To obtain all-female stocks, gynogenetic genotypic females should be masculinized to phenotypic males by androgen treatment. The use of the sperm of the resulting neomales to fertilize normal eggs would significantly increase the proportion of females in the offspring.

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CAPÍTULO IV
DISCUSIÓN GENERAL

DISCUSIÓN GENERAL

En este apartado se discuten brevemente algunas cuestiones adicionales relacionadas con los temas tratados en los capítulos precedentes. Aunque se ha hecho un esfuerzo al respecto, no se tiene la certeza de haber conseguido evitar una cierta repetición, repetición hasta cierto punto inevitable en mayor o menor grado al intentar contextualizar las cuestiones adicionales arriba aludidas.

4.1. LA FERTILIZACIÓN ARTIFICIAL EN LA APLICACIÓN DE LAS TÉCNICAS DE MANIPULACIÓN CROMOSÓMICA

La fertilización artificial utilizada habitualmente en la reproducción del rodaballo en cultivo intensivo facilitó la aplicación de las técnicas de manipulación cromosómica. En esta especie las puestas sólo se producen espontáneamente en grandes volúmenes de agua, y en cultivo intensivo normalmente los gametos de los machos (el esperma) y de las hembras (los huevos) se obtienen por medio de masaje abdominal. La fertilización se realiza más tarde, poniendo en contacto el esperma y los huevos y añadiendo agua de mar para activar el esperma. Este tipo de fertilización permitió la manipulación de los gametos y de los embriones.

En este estudio, el método utilizado para iniciar el proceso de inducción a la maduración y la puesta fue el habitual, mediante el cambio brusco del fotoperiodo desde 8h luz:16h oscuridad, a 16h luz:8h oscuridad (Forés et al., 1988). Esto desencadenó el inicio de las puestas de las hembras en los aproximadamente 60 días siguientes. Los huevos se extrajeron de las hembras y se mantuvieron en su propio fluido ovárico durante unos minutos, en baño maría a la temperatura del agua de los reproductores, sin aparente pérdida de calidad. Los machos produjeron esperma durante un amplio periodo de tiempo, empezando antes y terminando después de las puestas de las hembras. El rodaballo es una especie típicamente oligospérmica y, con el fin de solventar el problema del pequeño volumen obtenido en cada extracción, las fertilizaciones se realizaron con una mezcla de esperma de dos o tres machos, para

aumentar la capacidad de éxito de la fertilización e incrementar la variabilidad genética de la descendencia. En el caso de la inducción de la ginogénesis, en la que se debe usar esperma de un solo macho, el esperma se tuvo que diluir en solución de Ringer-200 frío (Chereguini et al., 1997).

Si se quiere mirar en positivo, el hecho de que el rodaballo prácticamente no ponga de forma espontánea en cautividad representó una gran ventaja en comparación con otras especies de peces en las que las puestas espontáneas dificultan la obtención del esperma y de los óvulos sin fecundar, lo que constituye un problema añadido en la aplicación de estas técnicas.

Así, las técnicas de inducción de la triploidía y de la ginogénesis, fueron relativamente fáciles de aplicar en el rodaballo, pero fueron también muy exigentes con respecto a la calidad de los gametos. El uso de gametos de baja calidad redujo la eficiencia de los procesos de manipulación genética, como ya ha sido descrito para otras especies (Lou y Purdon, 1984; Johnstone, 1985; Díaz et al., 1993).

4.2. LA TRIPLOIDIA EN EL RODABALLO

Inducción de la triploidía por choque térmico

La inducción de la triploidía en el rodaballo se realizó aplicando choques fríos a los huevos recién fertilizados según la técnica descrita para numerosas especies de agua dulce y marina, tomando como referencia el método desarrollado en la lubina descrito por Felip et al. (1997). Los valores óptimos de las variables fundamentales para la inducción de la triploidía (temperatura, momento de aplicación y duración del choque), son específicos de cada especie (Benfey, 1989; Cherfas et al., 1994) y en el rodaballo fueron: temperatura -1ºC-0ºC, el momento del choque, 6,5 minutos después de la fertilización y la duración, 25 minutos.

Los valores de temperatura y momento de aplicación del choque fueron similares a los utilizados en otras especies de peces teleósteos marinos incluyendo peces planos (Felip et al., 2001a), comenzando el choque en los primeros 15 minutos después de la fertilización y manteniendo la temperatura próxima a 0ºC. En el rodaballo, temperaturas más altas disminuyeron el porcentaje de triploidía y temperaturas más bajas, si bien aumentaron las tasas de triploidía, disminuyeron el porcentaje de supervivencia. La precisión del rango de temperatura (-1 y 0ºC), fue imprescindible paran obtener altas tasas de triploidía y supervivencia.

La duración del choque fue similar a la utilizada en otras especies de peces marinos cultivados a la misma temperatura entre 13-14ºC, como la lubina (Felip et al., 1997), pero bastante más cortos que los que se necesitaron en especies de peces planos cultivados a temperaturas muy bajas, como el fletán a 6ºC (Holmefjord y Refstie, 1997), o la platija a 7ºC (Lincoln, 1981), donde los choques tuvieron duración de 45 minutos a tres horas. Díaz et al. (1993) sugieren que la diferencia de temperatura entre el prechoque y el choque es más importante que la del propio choque, lo que se evidencia aquí cuando se hacen estas comparaciones.

Aplicando estos valores, las tasas de triploidía alcanzadas fueron en la mayoría de los casos del 90-100% (Piferrer et al., 2003). Estos valores fueron semejantes a los obtenidos para la lubina (*Dicentrarchus labrax*) (Felip et al., 1997), y mayores que los conseguidos en otras especies marinas tales como la dorada (*Sparus aurata*) (Garrido-Ramos et al., 1996) y el fletán (*Hippoglossus hipoglossus*) (Holmefjord y Refstie, 1997). En la manipulación de cantidades grandes de huevos (aprox. 300 ml), las tasas de supervivencia oscilaron entre el 60-70 % respecto de los controles no tratados, pero respecto a los controles *sham*, la supervivencia no fue significativamente diferente, lo que podría ser indicativo de que la disminución de la supervivencia fue más un problema de manipulación que de la triploidía en sí. Estas tasas son menores que las obtenidas a escala del laboratorio, lo que posiblemente esté relacionado con la mecánica que exige el manejo de tales cantidades de huevos.

El análisis de número de nucleolos por núcleo (NOR) utilizado en la determinación del nivel de ploidía en embriones y en larvas es una técnica barata y fácil de realizar, y ha sido utilizada en muchas especies de peces (Phillips et al., 1989; Felip et al., 1997). En el rodaballo, debido a la observación de polimorfismo en el número de NOR en un pequeño porcentaje de individuos, se cuantificó el nivel de error del método siendo menor del 5%, por lo que se determinó que el análisis de NOR es perfectamente válido para determinar la ploidía en rodaballo (Piferrer et al., 2000). En peces, la determinación del nivel de ploidía midiendo la longitud del eje mayor de los eritrocitos en una muestra de sangre, ha sido descrita en todas las especies en las que se ha inducido triploidía (Benfey, 1999), siendo un método fiable y el más usual. En el rodaballo, las muestras de sangre se obtuvieron fácilmente del arco branquial, sin necesidad de anestesiar al pez. Este método es un sistema de determinación del nivel de ploidía muy fácil, que permite analizar muchas muestras en poco tiempo y sin necesidad de sacrificar a los peces

Consecuencias biológicas de la triploidía

La incidencia de la inducción artificial de la triploidía en los peces depende de la especie y de la etapa de desarrollo del individuo. En el rodaballo, la triploidía indujo cuatro cambios fundamentales: 1) esterilidad en ambos sexos, 2) aumento de la proporción de hembras, 3) mayor crecimiento después de la maduración sexual, 4) tolerancia disminuida a niveles bajos de oxígeno.

En general, la triploidía induce esterilidad en los peces en todas las especies estudiadas (Benfey et al., 1989; Hussain et al., 1995; Benfey, 1999; Carrasco et al., 1999), y frecuentemente la incidencia de la esterilidad es mayor en hembras que en machos. Las hembras triploides de especies como la trucha arco iris (Nakamura et al., 1987), y de la lubina (Felip et al., 2001b), mostraron escaso o nulo desarrollo gonadal, mientras que los machos triploides de algunas especies como el salmón Atlántico (Refsie, 1984) y de trucha (Benfey et al., 1986) fueron capaces de desarrollar incluso caracteres sexuales secundarios, aunque sus gónadas fueron

anormales y reducidas. En otras especies, los machos pueden presentar actividad espermatogénica e incluso la producción de espermatozoides, aunque en pequeña cantidad y muy diluidos, y presentando anormalidades (Nakamura, et al., 1993), por lo que aunque genéticamente estériles, estos animales no son completamente infértiles.

En el rodaballo, la triploidía indujo esterilidad y también con distinta incidencia en los machos y en las hembras. En los machos la esterilidad fue gamética, y los testículos se desarrollaron de forma semejante a los diploides aunque alcanzaron menor tamaño, observándose incluso actividad espermatogénica, pero no se observaron espermatozoides. En las hembras, la esterilidad fue gonadal y los ovarios no se desarrollaron apareciendo muy pequeños y de aspecto rudimentario. Dunham, (2004), observó que hembras triploides fueron capaces de desarrollar oocitos maduros en especies como la trucha común (Smith y Benfey, 2000) y tilapia (Pandian y Varadaraj, 1988), a edades más avanzadas de aquellas en las cuales maduran las hembras diploides, pero en los rodaballos triploides los oocitos no evolucionaron más allá de estado previtelogénico al menos hasta los cuatro años de edad.

En las gónadas de hembras de rodaballo triploides se observó la presencia de células en apoptosis, no observadas en las hembras diploides. El proceso de apoptosis o muerte celular programada ha sido observado en células germinales femeninas de muchas especies, asociado a las primeras fases del desarrollo de la oogénesis y se cree que afectan a células anormales que debido a sus defectos no van a ser capaces de desarrollarse y madurar normalmente. El papel de la apoptosis en el control del desarrollo gonadal en peces todavía no es bien conocido (Callard et al., 1995; Wood y Van Der Kraak, 2001), aunque se sabe que la apoptosis tiene una función importante en la regulación de la gametogénesis y que está asociada con algunos problemas de infertilidad (Terrones et al., 2003).

En los rodaballos diploides la tasa de sexos normal es 1M:1H (Imsland et al., 1997; Iglesias, com. pers.) pero en este estudio la proporción de sexos en diploides

fue 1M: 0.6H, y en triploides fue 1M:3.3H. La mayor proporción de hembras observada en la progenie de rodaballos triploides puede ser causada por la distinta mortalidad entre sexos en estadios tempranos o puede estar relacionada con la doble dotación genómica materna que tienen estos peces (Benfey, 1999) ya que la progenie triploide recibe dos juegos de cromosomas de la madre y uno del padre. La alteración de la tasa de sexos en peces inducidos artificialmente a triploidía, ha sido observada en, por ejemplo, la tilapia (Penman et al., 1987; b; Mol et al., 1994), e incluso en algunas especies se observaron ausencia total de hembras, como en el pez cebra (Kavumpurath y Pandian, 1990) o en la dorada japonesa (Kitamura et al., 1991).

Así, a pesar de que el mayor porcentaje de hembras ha sido descrito en otras especies de peces inducidos a la triploidía, mientras las causas no se determinen con absoluta certeza o los experimentos de triploidía en el rodaballo se repitan en suficientes ocasiones, no se puede afirmar con rotundidad que la triploidía en el rodaballo sea la única razón de la tasa de hembras obtenido en este estudio. Además, no se puede descartar el efecto de la temperatura sobre la tasa de sexos, no documentado en esta especie.

Los rodaballos triploides fueron morfológicamente indistinguibles de sus homólogos diploides y, como ocurre en la mayoría de las especies de peces inducidas a la triploidía, el aumento de tamaño celular en si no produjo un mayor crecimiento de los peces, ya que normalmente el número de células es menor que en diploides (Thorgaard, 1983; Ihssen et al., 1990; Benfey, 1991, 1999). Durante la fase de juveniles, los rodaballos triploides tuvieron un crecimiento similar al de los diploides pero como en otras muchas especies la maduración sexual marcó un antes y un después en el modo de crecimiento. A partir de la maduración sexual, la tasa de crecimiento de los peces diploides estuvo modulado por los ciclos anuales de vida, con incrementos rápidos antes de las puestas debidos a un gran aumento del peso de sus gónadas, no interesante en animales destinados a producción de carne, seguido de pérdidas de lo ganado tras las mismas, siendo este efecto particularmente notorio en las hembras. En cambio en los triploides, debido a su esterilidad, el crecimiento fue regular y continuado.

En este estudio, la diferencia de peso observada entre los rodaballos diploides y triploides fue debida a dos factores: a la esterilidad inducida por la triploidía y a la mayor proporción de hembras de mayor crecimiento que los machos (Imsland et al., 1997), observada en este experimento, pero no se ha podido cuantificar que parte de esta diferencia de peso fue debida a la esterilidad y cual al mayor porcentaje de hembras, debido a la imposibilidad de sexar los rodaballos triploides vivos.

Resultados similares fueron observados en *Ictalurus punctatus* (Wolters et al.1982), *Pleuronectes platessa* (Lincoln, 1981) y en trucha arco iris (Thorgaard, 1986; Carrasco et al. 1999), mientras que en el salmón el crecimiento fue similar entre ambas ploidías (Benfey et al., 1989), y en la lubina fue menor desde los 3–4 años, pero alcanzó un tamaño similar cuando los peces alcanzaron 1 Kg de peso (Felip et al., 2001c).

En los rodaballos triploides el hematocrito y la concentración de hemoglobina son más bajos que en diploides (Cal et al., 2005). En un estudio preliminar (Cal et al., 2001) los animales toleraron mal los bajos niveles de oxigeno, demostrando una menor capacidad de resistencia a condiciones de hipoxia aguda que los diploides, quizás como resultado de una menor capacidad de utilización del oxígeno a bajas concentraciones. La información acerca de la tolerancia a bajos niveles de oxigeno de los peces triploides es contradictoria en las distintas especies (Dunham, 2004). Algunos autores indican que la baja tolerancia esta relacionada con la menor capacidad de la sangre de transportar el oxigeno, siendo la concentración de la hemoglobina un indicador de la habilidad de la sangre para transportar y distribuir el oxígeno a los tejidos.

La menor capacidad de los peces triploides para utilizar oxigeno en condiciones de alta demanda, y/o baja disponibilidad de oxígeno ha sido descrito frecuentemente en especies como el salmón Atlántico (Quillet and Gaigón, 1990; Sadler et al., 2000); el ayu (Aliah et al., 1991); la trucha arco iris (Ojolick et al., 1995), en un intento por encontrar las causas de las mortalidades que

esporádicamente se producen entre los triploides de diferentes especies en las granjas, pero hasta ahora no se ha podido demostrar que las mortalidades acaecidas lo hayan sido por asfixia.

La mortalidad observada en situaciones de hipoxia aguda quizás sea sólo el reflejo de una situación llevada al extremo de un problema habitual en el cultivo intensivo de los peces triploides y, aunque puede no ser un obstáculo para el cultivo de rodaballos triploides, sí puede esconder el menor bienestar de los peces y repercutir en el rendimiento del cultivo.

La triploidía en sistemas de producción

La inducción de la triploidía se presenta como una técnica fácil de aplicar en los sistemas de producción, ya que no requiere instalaciones especiales, siempre y cuando se utilicen gametos de buena calidad y las variables del choque (temperatura, momento del choque y duración del mismo) se apliquen con precisión. Para inducir triploidía a cantidades de huevos del orden de décimas de litro la temperatura del agua al inicio del choque debe estar por debajo de 0ºC sin llegar a congelarse, a fin de compensar el aumento de temperatura que se produce en el baño al introducir los huevos a 13-14ºC. Para ello es muy conveniente explotar el menor punto de congelación del agua salada con respecto al agua dulce. El agua salada a 35$^{o}/_{oo}$ de salinidad no se congela hasta los –1,3ºC aproximadamente, por lo que es factible trabajar a temperaturas iniciales de –1ºC. La inducción de la triploidía se consigue así con tasas del 95-100%, y con supervivencia entre 60-70% respecto del control no tratado. El éxito de la inducción se comprueba determinando el nivel de ploidía en una muestra de larvas de 1 ó 2 días de vida, mediante análisis del número de NOR, o más tarde en peces, en una muestra de sangre, midiendo el eje mayor de los eritrocitos en una extensión teñida o de forma automática determinando el volumen de los eritrocitos con un Coulter Counter. Esta técnica no requiere sacrificar ningún animal.

El cultivo de los triploides se realiza de la misma forma que el de los diploides, pero como han descrito problemas de competencia por el alimento cuando se cultivan juntos, es aconsejable cultivarlos por separado (Carter et al., 1994; Tave, 1993). Los rodaballos inducidos a triploidía son grupos de peces mayoritariamente hembras estériles, por lo que combinan las ventajas de ser hembras (que crecen más que los machos) y de no sufrir los efectos asociados a la maduración (ya que son estériles). La mayor proporción de hembras de mayor crecimiento y totalmente estériles, motivó que la biomasa generada en las unidades de cultivo de los triploides a los 4 años de edad fue del 14% más alta que la de los diploides. El hecho de ser mayoritariamente hembras y estériles, también eliminó dos factores importantes de dispersión de los tamaños, eliminando la necesidad de desdobles en las unidades de cultivo asociados muchas veces con problemas sanitarios. La esterilidad permite que los peces mantengan una calidad del producto constante y facilita el que se puedan comercializar en cualquier época del año, no produciéndose mortalidades asociadas a este periodo. Así pues la inducción a triploidía se presenta como una alternativa muy interesante para la obtención de rodaballos de 2 o más kilos.

4.3. LA GINOGÉNESIS EN EL RODABALLO

Inducción de la ginogénesis: el carácter oligospérmico del rodaballo y la fragilidad larvaria

La inducción de la ginogénesis en el rodaballo se realizó fertilizando los huevos con esperma cuyo DNA había sido previamente inactivado mediante radiación UV (Cal et al., 2001; Piferrer et al., 2004). La condición oligospérmica del rodaballo no evitó que la radiación tuviese que hacerse con esperma previamente diluido (1:10) en solución de Ringer-200 (Chereguini et al., 1997) para asegurar su inmovilización y conservación. La dosis de radiación UV óptima para producir el efecto Hertwig fue 30.000 $erg.mm^{-2}$, similar a la utilizada por Felip et al. (1999) en la lubina, lo que sugiere similitud en la relación dosis-efecto en estas dos especies marinas.

El efecto de la radiación de UV sobre la inactivación del DNA del esperma en el rodaballo fue muy variable entre los diferentes machos en función de algún factor de calidad no determinado e independientemente de la concentración inicial o de la dilución utilizada. Esto se reflejó en la gran variabilidad del éxito de los ensayos de inducción de ginogénesis, ya que cuando el esperma irradiado fue capaz de fertilizar, los embriones fueron todos o casi todos ginogenéticos, mientras que en otros casos no se produjo fertilización. Felip et al. (1999) sugirieron con respecto a la lubina que el motivo de la baja tasa de fertilización pudiera ser debida a la poca supervivencia o nula movilidad de espermatozoides tras la radiación, pero en el rodaballo espermatozoides con alta movilidad muchas veces no fertilizaron, y por el contrario otros con baja movilidad sí fueron capaces de fertilizar y producir embriones.

Los embriones obtenidos tras la fertilización con esperma irradiado fueron haploides y por lo tanto no viables, y presentaron los signos externos típicos de la condición haploide. En muchos casos sobrevivieron hasta los últimos estadios pero normalmente no eclosionaron, y cuando lo hicieron no sobrevivieron nunca más allá de las 24 horas siguientes.

La determinación de la herencia exclusivamente materna en los embriones y las larvas se realizó en todos los casos utilizando microsatélites (Castro et al., 2003). Los loci microsatélites usados han demostrado ser altamente variables en varias poblaciones estudiadas (Coughlan et al., 1996; Estoup et al., 1998; Bouza et al., 2002). Esta variabilidad genética permitió un alto nivel de confidencia como testigos de paternidad (Castro et al., 2003).

En el rodaballo, estas determinaciones resultaron sencillas, ya que al conocerse los padres utilizados en el cruzamiento debido a la fertilización artificial, fue suficiente analizar sólo dos loci diagnóstico cuidadosamente seleccionados en el descendiente para confirmar la herencia exclusivamente materna. La utilización de dos loci es recomendada ya que a veces se producen mutaciones en estos loci. Además, puede haber transmisión paterna residual en algunos descendientes.

(Thorgaard, 1985). Esta técnica ha demostrado ser muy fiable y además cuenta con la ventaja adicional de no tener que sacrificar al animal, ya que se necesita muy poca muestra para el análisis.

Consecuencias biológicas de la ginogénesis.

La inducción de la ginogénesis en el rodaballo produjo en la descendencia tres importantes consecuencias biológicas: 1) haploidía (n) y, por tanto, inviabilidad, 2) menor tasa de eclosión y de supervivencia durante el primer año de vida en los ginogenéticos diploides, 3) un aumento muy significativo de la proporción de hembras en los mismos.

Los embriones ginogenéticos obtenidos tras la activación del huevo con esperma de rodaballo irradiado fueron haploides y en consecuencia no viables. La viabilidad se consiguió restaurando la diploidía, evitando la extrusión del segundo corpúsculo polar, mediante la aplicación de un choque térmico frío al huevo recién fertilizado, de forma semejante a la descrita para inducir triploidía.

En los rodaballos ginogenéticos diploides se observó menor tasa de eclosión y de supervivencia durante el primer año de vida, como en la mayoría de las demás especies (Cherfas, 1981), probablemente debido a la expresión de alelos recesivos deletéreos como consecuencia de la mayor homocigosidad (Ihssen, et al., 1990; Leary et al., 1985) y a la fragilidad larvaria de esta especie (Planas y Cunha, 1999). Baynes et al. (2004) describen supervivencias entre 9-40% de ginogenéticos producidos mediante activación de huevos de rodaballos con esperma de fletán no irradiado.

En adultos hasta 36 meses, la supervivencia fue similar a los diploides control. Los rodaballos ginogenéticos se desarrollaron normalmente mostrando un crecimiento y un desarrollo gonadal casi normal como en otras especies tales como la carpa (Cherfas, 1975) o la lubina (Felip et al., 2002). En el desarrollo gonadal la única alteración observada fue el retraso en maduración en las hembras ginogenéticas

con respecto a las de grupo control. Ambos sexos produjeron gametos fértiles capaces de producir embriones y desarrollarse hasta larvas normales.

Respecto al crecimiento, aunque todos los peces estaban marcados y el seguimiento fue individualizado durante todo el experimento, únicamente se utilizó un tanque de ginogenéticos diploides y otro de control de diploides. En consecuencia, al no haber replicados de las unidades experimentales, no se pudo determinar si las diferencias observadas fueron debidas a la condición de ginogenéticos o al efecto tanque, por lo que las conclusiones sobre crecimiento hay que considerarlas con reservas.

En definitiva, se determinó que los peces ginogenéticos son capaces de sobrevivir hasta adultos con una tasa similar a los diploides del control, una vez han superado las primeras fases del desarrollo y que son capaces de crecer y reproducirse de forma esencialmente igual que los diploides, y esto es suficiente como punto de partida para la formación de lotes monosexo.

La proporción de sexos observada en los experimentos de ginogénesis en este estudio, fue (1M:3H) y (0M:1H). Proporciones semejantes a estas han sido observadas en numerosas especies inducidas a ginogénesis, en las que las hembras resultaron ser el sexo homogamético (Felip et al., 2001a). La mayor proporción de hembras fue también observada en rodaballos inducidos a triploidía, y no se puede descartar la posibilidad de que debido al choque térmico se haya producido una mortalidad selectiva en los machos de esta especie.

El mecanismo de determinación sexual en el rodaballo se discute al final de este capítulo.

La ginogénesis en los sistemas de producción

La inducción de la ginogénesis en el rodaballo, puede tener dos aplicaciones muy concretas en los sistemas de producción. En primer lugar es un método eficiente

para la producción de líneas endogámicas (Thorgaard, 1983; Yamazaki, 1983; Ihssen et al., 1990) y la explotación de heterosis a través de cruzamientos entre razas. En segundo lugar, porque representa un método potencial muy efectivo para iniciar el cultivo de lotes todo hembras que crecen mas que los machos (Imsland et al., 1997).

En los experimentos realizados en este estudio, el porcentaje de ginogenéticos obtenidos fue del 90-100% y las tasas de hembras muy altas, pero el fracaso de la fertilización con esperma irradiado también fue muy frecuente. Por lo tanto, en base a esta dificultad, junto con la menor tasa de eclosión y la menor tasa de supervivencia durante el primer año de vida, la idea de inducir rutinariamente ginogénesis para la producción directa de lotes con alta proporción o 100% hembras no resulta nada aconsejable.

Sin embargo, la posibilidad de producir rodaballos ginogenéticos diploides, aún en cantidades limitadas, para transformarlos seguidamente en neomachos y utilizarlos como reproductores donadores de esperma, es un punto interesante de arranque en la producción de lotes de rodaballos todo hembras. La incorporación al lote de reproductores de un lote pequeño de neomachos así producidos sería suficiente, ya que a partir de ellos futuros neomachos se podrían obtener, simplemente masculinizando, un pequeño porcentaje de la progenie de 100% hembras obtenidas.

La ginogénesis en la investigación genética básica

La proporción de sexos en los ginogenéticos diploides puede dar información relevante sobre el mecanismo de determinación sexual en cada especie. En el rodaballo la proporción de sexos normalmente es 1M:1H (Imsland et al., 1997), lo que sugiere que esta especie no es muy sensible a cambios ambientales. En este estudio, la proporción de sexos observada en ginogenéticos fue en un primer experimento 1M:3H y en un segundo experimento 0M:1H.

En base al resultado de la proporción de sexos en el primer experimento (1M:3H) se podría sugerir un mecanismo de determinación sexual ZW(♀)/ZZ(♂), ya que 1) asumiendo la viabilidad del genotipo WW que produciría un 50% de WW (hembras), y el resto ZZ (machos), y 2) asumiendo además cierta recombinación durante la meiosis, entonces algunos peces podrían ser ZW (hembras normales), con lo que la proporción de sexos se desviaría hacia un mayor número de hembras. La probabilidad de recombinación entre el segmento determinante del sexo y el centrómero es una posibilidad que ciertamente podría explicar el exceso de hembras. Sin embargo, en la mayoría de los casos ha sido demostrada la existencia de mecanismos que previenen recombinación en cromosomas sexuales, para evitar desviaciones en la proporción de sexos. Si esto no fuera así, los peces podrían sufrir recombinación en una alta proporción lo cual no parece probable. En nuestro caso, un alto porcentaje de hembras tendría que haber aparecido en el control y este no fue el caso. De hecho, en el control del primer experimento las hembras fueron menos del 50%.

En teoría, la progenie ginogenética de peces de especies XX/XY inducidas a ginogénesis deberían ser todo hembras, pero en la realidad es posible la aparición de un pequeño porcentaje de machos (Felip et al., 2001a). Considerando además el 100% de hembras obtenidas en el segundo experimento de ginogenéticos, se puede confiar en que el mecanismo de determinación sexual en rodaballo sea XX/XY, ya que en la inducción de ginogénesis en peces sólo se pueden obtener una progenie del 100% de ginogenéticas si y sólo si el mecanismo de determinación sexual es del tipo XX hembras / XY machos. Hasta el momento no hay ninguna cita en la bibliografía donde se describa la obtención de progenie de ginogenéticos 100% hembras en peces con un sistema de determinación que no sea XX/XY.

A la vista de estos resultados, creemos que como en otros peces planos, lo más probable es que en el rodaballo las hembras sean el sexo homogamético. Sin embargo, es probable que además otros factores como genes autosomales menores y/o factores medioambientales estén implicados en el mecanismo de determinación sexual del rodaballo ocasionando que la progenie ginogenética a veces no sea 100%

hembras. Los porcentajes de sexos en grupos de rodaballos inducidos a la triploidía, no se ajustaron perfectamente a un mecanismo clásico de XX/XY, lo que apoya la presencia de algún tipo de efecto genético aditivo sobre la determinación sexual

En el rodaballo, la inducción de la ginogénesis (sin la aplicación del choque térmico utilizado normalmente para restaurar la diploidía), produjo embriones que en la mayoría de los casos se desarrollaron hasta los últimos estadios. Estos embriones no fueron viables como corresponde a su condición de haploides, pero constituyeron una fuente de DNA muy útil que ha permitido el desarrollo de un mapa preliminar de ligamiento con AFLPs y microsatélites como marcadores (Fortes et al., 2004).

Los mapas de ligamiento entre marcadores estiman las distancias genéticas entre estos marcadores y se pueden utilizar para buscar asociaciones entre regiones del genoma y carácteres de interés productivo, o lo que es lo mismo, localizar QLT (quantitative trait locus) (Danzmann, et al., 2001; Sakamoto et al., 1999).

CONCLUSIONES

CONCLUSIONES

1. La inducción a la triploidía puede conseguirse sin dificultad en el rodaballo mediante la aplicación de choques fríos a los huevos recién fertilizados, con un incremento de la mortalidad que sólo es aparente durante los primeros días de vida. La temperatura del choque y el tiempo de su inicio es muy similar a lo descrito para otras especies de peces planos a los que se ha inducido la triploidía. Sin embargo, su duración es mucho más corta, reflejo de la mayor temperatura de fertilización en el rodaballo.

2. A diferencia de lo observado anteriormente en la mayoría de las especies examinadas, la triploidía en el rodaballo no sólo induce la esterilidad completa en hembras sino también en machos, indicando un bloqueo completo de la espermatogénesis.

3. El aumento observado en la proporción de hembras en poblaciones de rodaballos triploides sugiere que la presencia de un juego entero adicional de cromosomas de origen materno debe traer consigo cambios en la dosis de genes no epistáticos implicados en la determinación del sexo en esta especie.

4. La triploidía afecta a la mayoría de parámetros sanguíneos, tanto a la morfología de los eritrocitos como a sus principales índices hematológicos. El aumento de tamaño de los eritrocitos va acompañado de una descompensada reducción de su número con la consiguiente disminución del hematocrito. Por tanto, si bien los rodaballos triploides no presentan problemas en condiciones normales, podrían ver mermada su capacidad para soportar condiciones subóptimas de concentración de oxigeno.

5. La inducción de la ginogénesis en el rodaballo es posible pero se ve dificultada al tratarse de una especie oligospérmica. La concentración de

espermatozoides inicial no es determinante para predecir la respuesta a los efectos de la radiación con luz ultravioleta. Ello sugiere la presencia de otros factores (fisiológicos, bioquímicos, etc.), aún no descubiertos, que sí deben influir en dicha respuesta. Por otro lado, la exposición a cantidades progresivas de radiación ultravioleta produce en el rodaballo un efecto radiobiológico Hertwig típico.

6. A pesar de la prevalencia de un polimorfismo en el número de regiones organizadoras del nucleolo en el rodaballo, el cálculo de dicho número representa un método fiable a efectos prácticos para la determinación del nivel de ploidía a escala poblacional. Sin embargo, no es un procedimiento apropiado para la determinación de la ginogénesis. Para ello se utilizaron dos loci microsatélites específicos. Estos microsatélites permiten la identificación de rodaballos ginogenéticos con una alta precisión, confirmando su utilidad no sólo para el establecimiento de paternidades sino también para la determinación de la herencia exclusivamente materna.

7. Aparte del implícito aumento de la homocigosidad y la consabida pérdida de supervivencia en la primera generación, la inducción de la ginogénesis en el rodaballo se traduce en un desarrollo esencialmente normal, con unas gónadas capaces de completar la gametogénesis, aunque con cierto retraso, y producir gametos viables.

8. La obtención de poblaciones en las cuales todos los peces son hembras tras la inducción de la ginogénesis significa que en el rodaballo las hembras son el sexo homogamético. Sin embargo, la presencia ocasional de machos en otras indica la existencia de factores secundarios determinantes del sexo, la posibilidad de efectos del ambiente sobre dicha determinación, o ambas cosas a la vez.

9. La inducción de la triploidía no implica un mayor crecimiento en el rodaballo. Sin embargo, el considerable número de hembras -de mayor

crecimiento que los machos-, la menor dispersión de tallas y la menor o nula mortalidad tras la época de puestas observada en las poblaciones hace de la triploidía una interesante opción para la acuicultura del rodaballo, particularmente en la producción de peces de gran tamaño.

10. El cruzamiento de neomachos ginogenéticos con hembras normales recuperaría la heterocigosidad y permitiría la producción de poblaciones formadas exclusivamente por hembras de viabilidad normal, lo que sin duda sería beneficioso para el cultivo del rodaballo.

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